IMPERIAL AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

IMP. AGRI. RES. INST. NEW DELHI
28-6-38
LIBRARY

28-6-38
LIBRARY

THE JOURNAL
OF
BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED FOR THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

EDITORIAL BOARD

RUDOLPH J. ANDERSON
STANLEY R. BENEDICT
W. MANSFIELD CLARK
ELMER V. McCOLLUM
LAFAYETTE B. MENDEL
DONALD D. VAN SLYKE

VOLUME 106

BALTIMORE
1934

Copyright, 1934
by
THE JOURNAL OF BIOLOGICAL CHEMISTRY

PUBLISHED AT CORNELL UNIVERSITY MEDICAL COLLEGE FOR
THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.
WAVERLY PRESS, Inc.
Baltimore, U. S. A.

CONTENTS OF VOLUME 106

No. 1, August, 1934

PAGE

Pearson, P. B. Inorganic phosphorus of horse serum. The effect of age and nutrition 1

Robinson, Howard W., Price, J. Waide, and Cullen, Glenn E. Studies of the acid-base condition of blood. III. The value of pK′ in the Henderson-Hasselbalch equation for human and dog sera, determined with the Simms electrode 7

Halliday, Nellie. Further investigations concerning the new vitamin B growth-promoting factor for rats, found in whole wheat 29

Phillips, Paul H., Stare, F. J., and Elvehjem, C. A. A study of tissue respiration and certain reducing substances in chronic fluorosis and scurvy in the guinea pig 41

Goebel, Walther F., and Babers, Frank H. Derivatives of glucuronic acid. IV. The synthesis of α- and β-tetraacetylglucuronic acid methyl ester and of 1-chlorotriacetylglucuronic acid methyl ester 63

Elderfield, Robert C., and Rothen, Alexandre. Strophanthin. XXX. The ultra-violet absorption spectra of trianhydrostrophanthidin and trianhydroperiplogenin derivatives 71

Bernheim, Frederick, and Bernheim, Mary L. C. The oxidation of proline and alanine by certain tissues 79

Spielman, M. A. The chemistry of the lipids of tubercle bacilli. XXXIX. The constitution of tuberculostearic acid 87

Hilbert, Guido E., and Jansen, Eugene F. A study of the absorption spectra of some carotenoid pigments at liquid air temperatures and its applications to the carotenoid pigments of cow-pea leaves (*Vigna sinensis*) 97

Butler, Allan M., and MacKay, Eaton M. The influence of the sodium and potassium content of the diet upon the sodium concentration of human centrifuged red blood cells 107

Levene, P. A., and Tipson, R. Stuart. The partial synthesis of ribose nucleotides. I. Uridine 5-phosphoric acid 113

Katzman, Philip A., and Doisy, Edward A. The quantitative determination of small amounts of gonadotropic material 125

White, Julius. The synthesis of cystinyldiglycine and cystinyldialanine 141

Erickson, Betty Nims, Gulick, Margaret, Hunscher, Helen A., and Macy, Icie G. Human milk studies. XV. The non-protein nitrogen constituents 145

Laug, Edwin P. A reinvestigation of the phenomenon of a first acid change in whole blood 161

LEVENE, P. A., and MARKER, R. E. Symmetrical disubstituted methanes prepared from members of optically active homologous series of disubstituted carboxylic acids and their derivatives.... 173

PLATT, MURIEL E., and SCHROEDER, E. F. Glyoxalase. II. The distribution of glyoxalase in tissues of normal and cancerous albino rats.... 179

KIRK, ESBEN. Gasometric microdetermination of phosphoric acid... 191

KIRK, ESBEN, PAGE, IRVINE H., and VAN SLYKE, DONALD D. Gasometric microdetermination of lipids in plasma, blood cells, and tissues.... 203

BOSWORTH, A. W. Studies of the fat of human milk.... 235

McFARLANE, W. D. The distribution of iron in tissues, particularly liver, during peptic digestion and autolysis.... 245

CHAIKOFF, I. L., and KAPLAN, A. The blood lipids in completely depancreatized dogs maintained with insulin.... 267

BARBOUR, A. D. The effect of the saturated fatty acid content of the diet on the composition of the body fat.... 281

FISHER, A. M., and SCOTT, D. A. Peptic hydrolysis of insulin.... 289

FISHER, A. M., and SCOTT, D. A. The insulin content of the pancreas in cattle of various ages.... 305

FOLIN, OTTO. The preparation of sodium tungstate free from molybdate, together with a simplified process for the preparation of a correct uric acid reagent (and some comments).... 311

CHASE, BARBARA W., and LEWIS, HOWARD B. Comparative studies of the metabolism of amino acids. VI. The rate of absorption of leucine, valine, and their isomers from the gastrointestinal tract of the white rat.... 315

WILSON, WILLIAM R., and HANNER, JAMES P. Changes of total lipid and iodine number of blood fat in alimentary lipemia.... 323

MICHAELIS, L., and SCHUBERT, MAXWELL P. The reaction of iodoacetic acid on mercaptans and amines.... 331

KEIL, H. L., and NELSON, VICTOR E. The rôle of copper in carbohydrate metabolism.... 343

PILLSBURY, DONALD M., and KULCHAR, GEORGE V. The use of the Hagedorn-Jensen method in the determination of skin glucose... 351

HELLER, V. G., PAUL, HENRY, and THOMPSON, R. B. Changes in the blood calcium and phosphorus partition during the life cycle of the chicken.... 357

ALMQUIST, H. J., LORENZ, F. W., and BURMESTER, BEN R. Relation of depot fat to egg yolk fat in laying hens.... 365

SPIEGEL-ADOLF, MONA, and SEIBERT, FLORENCE B. Spectral analysis of purified tuberculin.... 373

ECK, JOHN C., and MARVEL, CARL S. A convenient synthesis of *dl*-lysine.... 387

JACOBS, WALTER A., and CRAIG, LYMAN C. The ergot alkaloids. III. On lysergic acid.... 393

DU VIGNEAUD, VINCENT, DYER, HELEN M., JONES, CHASE B., and PATTERSON, WILBUR I. The synthesis of pentocystine and homomethionine.... 401

BALLS, A. K., and SWENSON, T. L. The antitrypsin of egg white..... 409
LEVENE, P. A., and STILLER, ERIC T. Acetone derivatives of *d*-ribose. II..... 421

No. 2, SEPTEMBER, 1934

EVANS, HERBERT M., LEPKOVSKY, SAMUEL, and MURPHY, ELIZABETH A. Vital need of the body for certain unsaturated fatty acids. IV. Reproduction and lactation upon fat-free diets..... 431
EVANS, HERBERT M., LEPKOVSKY, SAMUEL, and MURPHY, ELIZABETH A. Vital need of the body for certain unsaturated fatty acids. V. Reproduction and lactation upon diets containing saturated fatty acids as their sole source of energy..... 441
EVANS, HERBERT M., LEPKOVSKY, SAMUEL, and MURPHY, ELIZABETH A. Vital need of the body for certain unsaturated fatty acids. VI. Male sterility on fat-free diets. Plate 1..... 445
BAERNSTEIN, HARRY D. A modification of the method for determining methionine in proteins..... 451
BLOCK, RICHARD J. The determination of the basic amino acids in small quantities of proteins by the silver precipitation method..... 457
SENDROY, JULIUS, JR., SEELIG, S., and VAN SLYKE, DONALD D. Studies of acidosis. XXII. Application of the Henderson-Hasselbalch equation to human urine..... 463
SENDROY, JULIUS, JR., SEELIG, S., and VAN SLYKE, DONALD D. Studies of acidosis. XXIII. The carbon dioxide tension and acid-base balance of human urine..... 479
KLEINER, ISRAEL S., and TAUBER, HENRY. Further studies on the zymogens of pepsin and rennin..... 501
WEIL, LEOPOLD, and RUSSELL, MARY A. A manometric micromethod for arginase determination. Enzymatic study of blood arginase in rats..... 505
BALL, ERIC G. Studies on oxidation-reduction. XXI. Phthiocol, the pigment of the human tubercle bacillus..... 515
YAVORSKY, MARTIN, ALMADEN, PHILLIP, and KING, C. G. The vitamin C content of human tissues..... 525
MORGAN, AGNES FAY, KIMMEL, LOUISE, THOMAS, RACHEL, and SAMISCH, ZDENKA. The effects of moderate doses of viosterol and of parathyroid extract upon rats..... 531
ROOT, RAYMOND W., and GREEN, ARDA ALDEN. The effect of acidity on the carbon monoxide-combining power of hemoglobin in the blood of marine fishes..... 545
KRUSE, H. D., SCHMIDT, MARGUERITE M., and MCCOLLUM, E. V. Studies on magnesium deficiency in animals. V. Changes in the mineral metabolism of animals following magnesium deprivation..... 553
ORENT, ELSA R., KRUSE, H. D., and MCCOLLUM, E. V. Studies on magnesium deficiency in animals. VI. Chemical changes in the bone, with associated blood changes, resulting from magnesium deprivation..... 573

Levene, P. A., and Schormüller, A. Synthesis of the phosphoric esters of hydroxyamino acids. III. Resolution of *d,l*-serinephosphoric acid and synthesis of *l*-hydroxyprolinephosphoric acid.... 595
Levene, P. A., and Tipson, R. Stuart. Monoacetone *d*-xyloketose. A preliminary note.... 603
Goddard, David R., and Michaelis, Leonor. A study on keratin... 605
Bauguess, Lyle C., and Berg, Clarence P. Tryptophane metabolism. VII. Growth and kynurenic acid production on amides of *l*-tryptophane.... 615
Pfiffner, J. J., Vars, Harry M., and Taylor, A. R. Extraction studies on the adrenal cortical hormone. I. Methods of preparation.... 625
Vars, Harry M., Taylor, A. R., and Pfiffner, J. J. Extraction studies on the adrenal cortical hormone. II. Yield from glands of various species.... 639
Pfiffner, J. J., and Vars, Harry M. Extraction studies on the adrenal cortical hormone. III. Distribution studies.... 645
Cutler, Jessie T. Studies on the carbohydrate metabolism of the goat. The blood sugar and the inorganic phosphate.... 653
Fruton, J. S., and Clarke, H. T. Chemical reactivity of cystine and its derivatives.... 667
Letonoff, T. V. A zinc hydroxide powder for the preparation of protein-free filtrates of blood.... 693
Jones, James H. The influence of the removal of the parathyroid glands on the development of rickets in rats.... 701
Jones, D. Breese, and Gersdorff, Charles E. F. Studies on digestibility of proteins *in vitro*. VI. Some partial cleavage products from peptic digests of casein.... 707
Kurtz, Floyd E., Jamieson, G. S., and Holm, George E. The lipids of milk. I. The fatty acids of the lecithin-cephalin fraction.... 717
Robbins, Benjamin H., and Lamson, Paul D. Further studies on the proteolytic enzyme content of latex from the fig and related trees.... 725
Ten Broeck, Carl. The differentiation of trypsins by means of the anaphylactic test.... 729
Schultze, M. O., Elvehjem, C. A., and Hart, E. B. The availability of copper in various compounds as a supplement to iron in hemoglobin formation.... 735
Schoenheimer, Rudolf, and Sperry, Warren M. A micromethod for the determination of free and combined cholesterol.... 745
Levene, P. A., and Muskat, Irving E. The synthesis of theophylline-5-methyl-rhamnofuranoside.... 761
Niemann, Carl, and Link, Karl Paul. Synthesis of the hexuronic acids. VII. The synthesis of *l*-glucuronic acid and the resolution of *dl*-galacturonic acid.... 773
Donal, John S., Jr. An ultramicromethod for the determination of the oxygen content of blood.... 783
Index to Volume 106.... 799

INORGANIC PHOSPHORUS OF HORSE SERUM

THE EFFECT OF AGE AND NUTRITION

By P. B. PEARSON

(*From the W. K. Kellogg Institute of Animal Husbandry, University of California, Pomona*)

(Received for publication, April 9, 1934)

Relatively little information is available on the phosphorus content of horse blood. Abderhalden (1), in his pioneer investigations of 1898, analyzed the blood of two horses and reported 3.3 mg. of inorganic phosphorus per 100 ml. of serum. Robinson (2), using the method of Fiske and Subbarow, determined the inorganic phosphorus content of the blood of forty-four horses and found a range of 1.92 to 5.36 mg. per 100 ml. of serum with an average of 3.19, but did not indicate the age or breed of the animals from which the blood samples were obtained. The most complete work on the normal phosphorus values for mature horses is that of Kintner and Holt (3), who analyzed the blood of 69 horses and found a range of 2.63 to 5.00 mg. of inorganic phosphorus per 100 ml. of serum with an average of 3.55 mg. In a recent paper Dimock and Healy (4) report an average of 4.9 mg. of inorganic phosphorus per 100 ml. of serum obtained from four thoroughbred yearling fillies. They observed no significant change in the phosphorus content of the serum from overfed fillies that received, in addition to the standard diet, haliver oil and ultra-violet ray treatments.

Methods

The blood samples were obtained from 76 horses, varying in age from 118 days to 23 years. All of the animals used for the study of the normal phosphorus values had been on a standard diet for several months. This diet consisted of a limited amount of grain and green feed and a liberal allowance of good quality hay. Those under 10 months of age were not yet weaned. Mature horses grazed on a pasture devoid of green herbage were observed in order to ascertain if this nutritional régime affected the serum phosphorus. Except for three grade Arabians, all of the animals were pure-bred Arabian horses of similar lines of breeding. Consequently, the genetic variability, if such occurs, should be a minimum factor.

The blood was drawn from the jugular vein and the serum from the individual samples was separated from the corpuscles by centrifugation. The inorganic phosphorus was determined by the method of Youngburg and Youngburg (5). At frequent intervals simultaneous determinations were also made by utilizing the method of Fiske and Subbarow (6). This procedure served as a double check.

Normal Phosphorus Values—For the purpose of statistical analyses of the data, the animals are divided into age groups by years up to the time they reach maturity, when a more or less constant level for inorganic phosphorus of the serum is reached. The mean concentration of the inorganic phosphorus of the serum is recorded in Table I.

TABLE I

Mean Concentration of Inorganic Phosphorus of Serum

No. of animals	Age	Inorganic P per 100 ml. serum	Standard deviation
	yrs.	*mg.*	
17	Under 1	5.23 ± 0.130	0.536
12	1–2	4.41 ± 0.092	0.320
7	2–3	4.29 ± 0.146	0.386
6	3–4	3.64 ± 0.141	0.347
18	Over 4	3.37 ± 0.041	0.176

The mean value of 3.37 ± 0.041 mg. of inorganic phosphorus per 100 ml. of serum for mature animals is not significantly different from the average of 3.55 mg. reported by Kintner and Holt (3). The mean value of 4.41 ± 0.092 mg. of inorganic phosphorus per 100 ml. of serum for animals 1 to 2 years old is somewhat lower than the value of 4.9 mg. reported by Dimock and Healy (4) for horses of similar age but of a different breed.

Correlation between Phosphorus and Age of Growing Animal—Since the inorganic phosphorus content of the serum decreases with increasing age of the growing animal, a correlation study was made between these two factors. The data used in this computation represent the inorganic phosphorus values ranging from 3.16 to 6.20 mg. per 100 ml. of serum obtained from forty animals ranging in age from 118 days to 3 years and 10 months. By means of the equation of Wallace and Snedecor (7) for machine calculation of the correlation coefficient, we find that the derived $r \doteq -0.768 \pm 0.066$. This high negative correlation means that with increasing age of the growing animal there is a decrease in the inorganic phosphorus content of the serum.

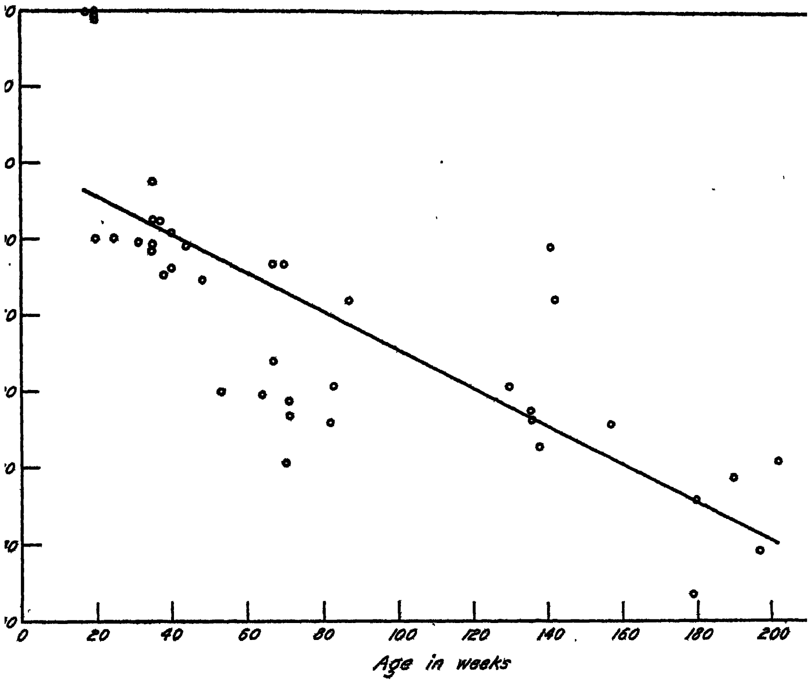


FIG. 1. Scatter diagram of relation between inorganic phosphorus content of the serum and age of the growing animal. The regression line shows the estimated or average phosphorus values corresponding to varying ages.

The regression line (Fig. 1) superimposed upon a familiar scatter diagram of the original data shows the estimated or average phosphorus values corresponding to varying ages. The regression line is immediately useful for estimating the inorganic phosphorus content of the serum of other horses within the age range considered, and with similar breeding and environmental conditions. The estimated value may also serve as a standard of comparison for inorganic phosphorus values observed in growing horses subjected to abnormal environmental conditions, nutritional régimes, or in pathological cases.

Effect of Nutritional Régimes—During the summer and autumn months sixteen mature horses were grazed on hill pasture. Since the precipitation during the 4 months prior to executing this study was nil, the feed consisted almost entirely of dry forage plants. The inorganic phosphorus of the serum of the horses grazed on this pasture ranged from 2.45 to 2.91 mg. per 100 ml. of serum, with a mean of 2.63 ± 0.054; standard deviation 0.217. The animals grazed on dry forage showed markedly lower phosphorus values than the mature horses receiving an adequate diet and having a mean serum phosphorus of 3.37 ± 0.041 mg.; standard deviation 0.176. These differences are highly significant statistically and leave no doubt as to the effect of the nutritional régimes on the inorganic phosphorus content of the serum.

In order to ascertain if the hypophosphoremia[1] was due to quantitative dietary differences, the sixteen horses were moved to an adjoining pasture with similar herbage not previously grazed during the year, as the former pasture had been, thereby insuring an abundant supply of feed. 14 days later blood samples were obtained from eight of the horses; the analyses showed no increment in the inorganic phosphorus content of the serum. This fact led us to suspect that the hypophosphoremia was probably due to the low phosphorus content of the forage plants on which the horses were grazed. The phosphorus and calcium contents of the three predominating species of forage plants gathered from the ungrazed pasture at the time the horses were moved into it are reported in Table II.

Inspection of Table II shows that both of the wild oats species are considerably lower in phosphorus and calcium than is bur-clover. The bur-clover was less abundant than either of the wild oat species. A comparison of the data in Table II with a large number of analyses of the same species gathered from native pastures in various sections of California, shows that the phosphorus values reported in Table II are more nearly comparable to the lower figures reported by Hart *et al.* (8), rather than with the normal values. The data indicate that the hypophosphoremia was due to the low phosphorus content of the herbage upon which the horses were grazed.

---

[1] The term hypophosphoremia is used to denote an inorganic phosphorus content of the serum significantly below the normal.

Recovery from Hypophosphoremia—Some indication as to the rapidity of recovery from the hypophosphoremia when the horses were changed to a different nutritional régime is obtained in the case of two animals. Two mature horses, selected at random from the sixteen head, were moved from the pasture and given a ration of 4 pounds of grain daily and a liberal allowance of good quality alfalfa and oat hay. When this change was made the inorganic phosphorus content of the serum was 2.45 and 2.91 mg. per 100 ml. respectively. 7 days later the inorganic phosphorus content of the serum of the first animal showed an increment from 2.91 to 3.29 mg. per 100 ml.; while 8 days later the second animal showed an increment from 2.45 to 3.33 mg. per 100 ml. of serum.

TABLE II

*Percentage Composition of Forage Plants**

Species	Moisture	Calcium	Phosphorus	Ratio, Ca:P:1
Wild oats (*Avena fatua*)	4.45	0.46	0.17	2.6
Slender wild oats (*Avena barbata*)	4.74	0.39	0.09	4.7
Bur-clover (*Medicago hispida*)	5.65	1.62	0.26	6.2

* Analyses for Ca and P on moisture- and silica-free basis.

SUMMARY

1. There is a high negative correlation ($r = -0.768 \pm 0.066$) between the age of the growing horse and the inorganic phosphorus content of the serum.

2. The inorganic phosphorus of the serum of the horse tends to approach a constant level at maturity. The level is, however, affected by an inadequate phosphorus intake.

3. Mature horses grazed on dry forage showed an inorganic phosphorus content of the serum significantly lower than that of horses receiving a limited amount of grain and green feed, and a liberal allowance of hay.

4. Recovery from the hypophosphoremia was effected within 8 days after changing from a phosphorus-deficient to an adequate phosphorus intake.

Acknowledgment is made to Mr. H. H. Reese for providing facilities and assistance for observations in the field.

BIBLIOGRAPHY

1. Abderhalden, E., *Z. physiol. Chem.*, **25**, 65 (1898).
2. Robinson, W. G., *Cambridge Univ., Inst. Animal Path., Rep. Director, III Rep.*, 156 (1933).
3. Kintner, J. H., and Holt, R. L., *Philippine J. Sc.*, **49**, 1 (1932).
4. Dimock, W. W., and Healy, D. J., *J. Am. Vet. Med. Assn.*, **83**, 806 (1933).
5. Youngburg, G. E., and Youngburg, M. V., *J. Lab. and Clin. Med.*, **16**, 158 (1930).
6. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
7. Wallace, H. A., and Snedecor, G. W., *Iowa State Coll., Off. Pub. 30*, No. 4, revised edition, 8 (1931).
8. Hart, G. H., Guilbert, H. R., and Goss, H., *California Agric. Exp. Stat. Bull. 543*, 10 (1932).

STUDIES OF THE ACID-BASE CONDITION OF BLOOD

III. THE VALUE OF pK′ IN THE HENDERSON-HASSELBALCH EQUATION FOR HUMAN AND DOG SERA, DETERMINED WITH THE SIMMS ELECTRODE*

By HOWARD W. ROBINSON, J. WAIDE PRICE, and
GLENN E. CULLEN

(From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati)

(Received for publication, March 22, 1934)

For many reasons well summarized by Peters and Van Slyke (1931), it is generally recognized that in studies of the acid-base equilibria in blood and other body fluids, the relations between pH and the concentrations of carbonic acid and bicarbonate are of fundamental importance.

The fundamental mass action equation for the system of a weak acid in the presence of its salts has been used, for the conditions existing in blood serum, in the form known as the Henderson-Hasselbalch equation

$$\text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

In this equation [BHCO$_3$] is the concentration of bicarbonate (B indicating monovalent cations) and is used as the equivalent of [HCO$_3^-$] in the fundamental equation. [H$_2$CO$_3$], the term for the concentration of carbonic acid, includes all free CO$_2$ and is calculated from the CO$_2$ tension, p, and the solubility coefficient, α; thus $[\text{H}_2\text{CO}_3] = \alpha p\text{CO}_2$. In the first development of this equation the constant pK′ was a summation constant which included the first dissociation constant of carbonic acid, the degree of dissociation of the bicarbonate molecules, and the assumption that the total concentration of H$_2$CO$_3$ could be used for the undissociated acid. With the introduction of the activity concept by Lewis, it was realized that the pK′ in this equation is a function of the activity coefficients of the ions which are in turn influenced by the thermodynamic environment. The pK′ in any dilute solution changes inversely with change in ionic strength. In serum the stability of pK′ will depend chiefly on the constancy of the total electrolyte and protein concentration. In both normal and pathological sera the ionic strength is relatively constant; *i.e.*, equivalent to a total monovalent salt concentration of 0.15 to 0.16 N.

---

* A preliminary report was presented before the American Society of Biological Chemists at Cincinnati, April, 1933 (*J. Biol. Chem.*, **100**, lxxxii (1933)).

Several points in the use of the Henderson-Hasselbalch equation in blood studies should be noted. (1) It is, of course, somewhat limited in its application, but is satisfactory for most pathological purposes (see Van Slyke (1922) for detailed discussion of this point). (2) It is preferable to retain the notation pH and pK′ rather than the more modern p*a*H and p*a*K′. (See discussion under calculation of pH.) (3) It is generally recognized that acid-base studies of blood can best be made with serum. (4) The pK′ value for acid-base studies is conventionally accepted as the value at 38°—"normal" human body temperature.

For several reasons we have thought it desirable to redetermine the value of this important constant, pK′, for human and dog sera: (1) The results of Cullen, Keeler, and Robinson (1925) and of Hastings, Sendroy, and Van Slyke (1928), although carried out with almost identical technique, are not in as close agreement as the accuracy of the technique warrants, and, moreover, in each set of determinations the results have too wide a spread and are too few in number for statistical analysis; (2) the influence of various pathological conditions on the serum pK′ needed more study; and (3) much of the experimental work in acid-base equilibria is based on dog sera, and, although the few comparisons available (Cullen, Keeler, and Robinson) indicate that the pK′ values for human and dog sera are identical, a more extensive comparison seemed desirable.

Sources of Error in pK′ Determinations

The method of determining the pK′ used in this work involves the equilibration (or saturation) of the serum with a gas mixture of known CO_2 content. Three analyses are required: (1) the determination of the total CO_2 of the equilibrated solution; (2) the analysis of the gas phase for CO_2 concentration; and (3) the determination of the pH.

The error in total CO_2 determination with the 50 cc. form of the Van Slyke manometric apparatus is within 0.3 volume per cent of CO_2. For the average serum CO_2 content this corresponds to about 0.003 pK′.

The errors which have undoubtedly caused most of the variation in previous reports are concerned with the CO_2 tension, which is involved both in the saturation of the serum with CO_2 and in the pH determination with the hydrogen electrode. The H_2CO_3 value of the saturated serum, calculated from the CO_2 tension, depends upon the accuracy of the tension adjustment, or analysis of the final CO_2 concentration, and upon the constancy of the solubility coefficient. This coefficient has been carefully redetermined for 38° by Van Slyke, Sendroy, Hastings, and Neill (1928) and found to have a value in normal human serum of 0.510 with variations in the third decimal place. They found that, in pathological serum containing much fat, the value of this coefficient may reach 0.540. Van Slyke, Sendroy, and Liu (1932) state in regard to serum high in fat, "However, even in such serum, it is probable that the value 0.510 represents approximately the solubility of CO_2 in the *water phase*, and that the use of the value, $\alpha = 0.51$, in calculating $[H_2CO_3]$, seldom involves an error of over 1 per cent." This would influence the pK′ value only 0.004 unit. In the present work we have assumed that the value of the solubility coefficient for dog serum is the same as that for human serum. Since a constant figure for 38° is used, any changes in the actual solubility would be undetected as such but would influence the value of pK′.

For the pH measurement, with any hydrogen electrode, the greatest accuracy is obtainable only when the CO_2 tension of the CO_2-H_2 gas mixture matches exactly the CO_2 tension of the saturated serum. Under such conditions the pH determination in serum is accurate to ± 0.005 pH. The Hasselbalch refill technique, by which the CO_2-H_2 gas mixture *in* the electrode is brought, by successive refills with serum, to the same CO_2 tension as that of the serum, as evidenced by the attainment of a constant potential, is satisfactory only if enough serum is available for the many refills usually required. The supply of human serum is often too limited for satisfactory pH determinations with the refill technique.

In previous work on pK′, both in our laboratories and in Van Slyke's, a separate CO_2-H_2 mixture of the same CO_2 tension used for equilibrating the serum has been prepared for the hydrogen electrode pH determination. This means that two separate CO_2 gas mixtures have to be prepared and thus the possibility of error is doubled.

In order to avoid this and to utilize a known constant CO_2-H_2 mixture, we have returned for this study to the principle, used by Hasselbalch (1910) in his original work and later by Warburg (1922), of utilizing the *same* CO_2-H_2 mixture in one apparatus for both saturation and for the pH determination. The Simms (1923) bubbling electrode proved itself (see below) admirably suited for this purpose, and has the added advantage of great economy of serum.

It should be pointed out that there is one further difference between the Hasselbalch technique of equilibration with CO_2-H_2 mixtures and the procedure used previously in Van Slyke's and in our laboratories, in that, in the latter method, the initial equilibrations of the sera were made with CO_2-air mixtures. Theoretically, the former should be better for the pH determination, since it avoids the introduction into the electrode of the small amounts of oxygen dissolved in the serum. If there is a difference in any changes which may occur in the serum during the equilibration under aerobic or anaerobic conditions, it is at present undetected.

Control of Methods and Technique

Total CO_2 Determinations—These were carried out in the Van Slyke 50 cc. manometric apparatus with Ostwald-Van Slyke "between marks" pipettes. All the determinations were made on 1 cc. samples. All the values for CO_2 in Tables I to III are for total CO_2, indicated by the symbol $[CO_2]$.

CO_2 Analysis of Gas Mixtures—These were made with the Haldane-Henderson gas analysis apparatus.

pH Determinations—These were made with the Clark electrode and with the Simms electrode (see below). The Clark electrode system (1915), in which the small cells with thermometer were used (Cullen, 1922), was set up in an air thermostat at 38° ± 0.02°.

The platinum electrodes for both the Clark and Simms apparatus were the plate type of sheet platinum about 2 × 3 mm. welded to a platinum lead-in wire which extended through the glass tube for connection with the lead to the potentiometer. Mercury was never used. The glass tubes for the Simms electrode have three small glass projections, about 1.5 cm. above the plate, to aid in centering without damaging the electrode coating. This coating was platinum black deposited from Merck's chloroplatinic acid to which a trace of lead chloride was added. The electrodes were freshly prepared and standardized for each serum determination as described in our previous work on pK′ (Cullen, Keeler, and Robinson).

Hydrogen and Hydrogen-CO_2 Mixtures—The hydrogen was that prepared electrolytically and furnished in high pressure cylinders by the Ohio Chemical Company. It was as near 100 per cent H_2 as could be determined by analysis. For the major portion of the pK′ work, mixtures of hydrogen and carbon dioxide prepared for us by this company have been used. Repeated analysis over a period of 3 years on one of these cylinders gave 5.56 ± 0.01 per cent CO_2.

Mixtures of hydrogen and carbon dioxide for comparison with those from the cylinders and for saturation of sera at varying CO_2 tensions were prepared with CO_2 produced from limestone in a Kipp generator. The gases were measured approximately by pressure (Van Slyke, Wu, and McLean, 1923) into a 3 liter Pyrex tonometer which contained about 50 cc. of a calcium chloride solution of 1.40 specific gravity. The solubility of CO_2 in this solution is 1/16 that of water. The tonometer was shaken vigorously, the $CaCl_2$ solution serving as a medium to mix the gases. It was then connected to a reservoir of the $CaCl_2$ solution which kept the gas mixture under sufficient positive pressure for transfer to the equilibration tonometer or for bubbling through the Simms electrode. In either case the residue of the gas mixture was run into two Bailey sampling bottles for duplicate gas analyses. This technique insures a gas sample for analysis which correctly represents the CO_2 tension of the gas phase with which the solution was equilibrated.

Equilibration of Sera with CO_2 Mixtures in Tonometers—This technique was in general that described by Austin *et al.* (1922) except that in all cases the final CO_2 tension of the gas phase was checked by analysis. In brief, the serum was transferred to the Pyrex tonometer, air was evacuated, and the tonometer refilled with the desired CO_2-H_2 mixture. The tonometer was rotated in the air bath for 15 minutes and then, always in the constant temperature bath, was connected to the CO_2-H_2 mixture which was allowed to wash through the tonometer, over the serum, for 10 or more minutes. The CO_2-H_2 mixtures were brought to the temperature and vapor pressure of the tonometer by washing through an elaborate 0.9 per cent saline washing train which was also in the 38° air bath. This alternation of washing and rotation was continued until equilibration was complete as evidenced by the agreement both of the successive total CO_2 determinations which were made after each rotation and of the analysis of the gas phase of the tonometer with that of the CO_2-H_2 mixture of the train. Usually two and always three saturations were sufficient. For all analyses the serum was removed to the familiar mercury sampling tubes (Austin *et al.*).

Calculations—The pH was calculated by substituting in the usual equation, pH = (E.M.F. − *e*)/0.0617, the electromotive force reading of the customary gas chain. The comparison electrode was a saturated KCl calomel half-cell. The value *e* was obtained with our standard reference solution, which is 0.1 N HCl prepared from constant boiling acid to which we assign the value pH = 1.080 (Cullen, 1922). The validity for this standard, which is now widely used in American biological laboratories, has been discussed previously in detail (Cullen, Keeler, and Robinson, 1925; Robinson, 1929). The potential of the system

$$^{-}\mathrm{Pt},\ \mathrm{H_2}\ (1\ \text{atmosphere})\ \Big|\ 0.1\ \mathrm{N\ HCl}\ \Big\|\ \mathrm{KCl_{(sat.)}}\ \Big\|\ \begin{matrix}\mathrm{KCl_{(sat.)}}\\ \mathrm{HgCl}\end{matrix},\ \mathrm{HgCl_{(s)}}\ \Big|\ \mathrm{Hg}^{+}$$

corrected to 760 mm. of dry hydrogen at 38° is 0.3024 volt. This makes the potential of the saturated calomel half-cell 0.2358 volt. The working standard of M/15 phosphate solution made from Merck's Blue Label "Sørensen" salts is almost as reproducible as the 0.1 N HCl. The M/15 solution of 8 parts of Na_2HPO_4 solution to 2 parts of KH_2PO_4 solution has a pH at 38° of 7.363.

In retaining the pH notation it is important to emphasize that the pH values used here for the calculation of pK′ of carbonic acid are identical with those previously reported by ourselves, with the pH values of Hastings and Sendroy (1924), the p*a*H values of Hastings and Sendroy (1925), the p*a*H values of Hastings, Murray, and Sendroy (1926–27), and the p*a*H values of Stadie and Hawes (1928, *a*); but are not identical with the p*a*H values of Sørensen and Linderstrøm-Lang (1924).

The CO_2 tensions were calculated from the per cent CO_2 in the gas phase. The relation for 38° is $pCO_2 = \text{per cent } CO_2 (B - 49.3)$ where B is the corrected barometric pressure at the time of the determination and 49.3 is the value of the vapor pressure of water (both in mm. of Hg).

The Van Slyke, Sendroy, Hastings, and Neill value for the solubility coefficient of CO_2 for serum at 38° of 0.510 was used throughout. In terms of volume per cent then

$$[H_2CO_3] = \frac{100}{760} \alpha pCO_2 = 0.0671\, pCO_2$$

The total CO_2 was calculated with Van Slyke-Sendroy (1927) factors. All determinations on sera equilibrated in the Simms electrode were multiplied by 1.01 to correct for the octyl alcohol dilution error (see below).

The CO_2 determinations in this paper are expressed in volume per cent; *i.e.*, cc. of gas, reduced to 0° at 760 mm., per 100 cc. of solution. They may be expressed in mM per liter by multiplying by 10 and dividing by 22.26, the volume of 1 mole of CO_2 at 0°, 760 mm.

The pK′ values were calculated, on the basis of the above factors, with the Henderson-Hasselbalch equation arranged in the form

$$pK' = pH_{e_{38^\circ}} - \log \frac{[\text{total } CO_2] - 0.0671\, pCO_2}{0.0671\, pCO_2}$$

The Simms Bubbling Electrode—For the reasons discussed under "Sources of error" we desired to saturate serum in the same vessel and with the same gas mixture that was to be used for the pH determination. The form of bubbling electrode described by Simms seemed suitable because of its simple temperature control and because its convenient 4-way stop-cock appeared to offer adequate protection against contamination from the saturated KCl solution used as the bridge to the calomel half-cell.

Since it has not been generally used for serum (Stadie, O'Brien, and Laug (1931) report its use, without details, in standardizing their glass electrode system), we felt it necessary to study carefully its suitability.

The accuracy of the temperature control was studied first. The Simms electrode has water jackets about the electrode vessel, the water saturating chamber, and the calomel half-cell. Water at constant temperature from a temperature-controlled bath, in this work a 5 gallon crock with toluene regulator and 250 watt lamps for heating, is circulated through the system by an electrically driven rotary pump. All leads to the water jacket are thick rubber tubing. With the water in the bath at 38.3°, the temperature of the fluid in the electrode vessel can be maintained at 38° ± 0.05°.

It is necessary to bubble the gas, hydrogen, or hydrogen-CO_2 mixture through the system at a constant rate which can be controlled best by a combination of reducing and needle valves attached to the high pressure gas tanks. The saturated calomel half-cells were always kept at 38° in an air thermostat whenever the system was not in use.

The electrode chambers of our vessels are slightly larger than the ones described by Simms and, with electrode tubes of 3.5 mm. diameter, allow equilibration of as much as 4 cc. of solution.

Comparison of Determinations with the Clark and the Simms Electrodes—Since, in biological work in this country, most of the pH and pK′ values have been determined with the Clark electrode, the performance of the Simms electrode was compared with that of the Clark electrode. The pH values of numerous phosphate solutions, determined in parallel with the two electrodes, always agreed within 0.002 to 0.003 pH. Likewise, the pH values of both human and dog sera, when equilibrated with CO_2-H_2 gas mixtures as detailed below, were identical with the two electrodes.

As a further control, not only of the identity of the results with the two types of electrodes, but of our technique in general, parallel pH and pK′ determinations were made with the Simms and the Clark electrodes on $NaHCO_3$-HCO_3-NaCl systems. CO_2-H_2 gas mixtures of known composition were equilibrated with solutions containing 0.03 M Na_2CO_3 and varying concentrations of NaCl. The resulting pK′ values agree approximately with values calculated with the equation of Hastings and Sendroy (1925) for this system, $pK' = 6.33 - 0.5\sqrt{\mu}$. Some of our mixtures were exactly the same as those used by Stadie and Hawes (1928, *b*). As our figures check so closely the work of these workers, it seems unnecessary to use space to record these experiments. The results with the two types of electrodes were identical.

We confirm Simms in that one of the advantages of this electrode is the freedom from contamination with KCl on successive equilibrations. Simms also reported constancy of reading to within a few tenths of a millivolt within 2 to 4 minutes. Even with phosphate solution, we require 15 minutes. Simms used a spiral platinum wire; we prefer the plate because of the ease of cleaning. This may be the cause for our slower equilibration.

Effect of Octyl Alcohol on pH and Total CO_2 Determinations—Serum froths so much in the narrow chamber of the bubbling electrode that some antifrothing agent is necessary. The pure octyl alcohol (Eastman's) used for this purpose had no influence on the pH determinations of phosphate-, carbonate-, or protein-containing solutions such as serum. This fact has long been known (Schmidt, 1916; Clark, 1928, p. 440) but is reaffirmed in view of the observation we report here that octyl alcohol may affect the total CO_2 determination.

We had satisfied ourselves that the pH of sera equilibrated in the bubbling electrode was the same as that determined in the Clark electrode on sera equilibrated in tonometers. However, when we analyzed these same sera, we were surprised to find that the total CO_2 content of sera from the Simms electrode was consistently about 0.6 volume per cent of total CO_2 *less* than those from the tonometers. After considerable speculation and experimenting, it dawned upon us that this was probably a *dilution effect* of octyl alcohol. The octyl alcohol is so thoroughly emulsified with the serum in the bubbling electrode that it remains as a uniform suspension for some time. Therefore, the 1 cc. sample used for the total CO_2 determination is a mixture of serum and octyl alcohol. Fortunately, we had used constant quantities, 3.6 cc. of serum and 0.04 cc. of octyl alcohol, for all determinations. With the sera averaging 55 volumes per cent of CO_2 this error of 1.13 per cent would mean 0.6 volume per cent less total CO_2. This point is so important in all such studies that several experiments with varying amounts of octyl alcohol were carried out. Of course, all the errors in the determination (0.2 to 0.3 volume per cent) are thrown into this factor, but the average of many determinations coincides with the calculated difference until so much alcohol was used, about 0.08 cc. per 3.6 cc. of sample, that it was no longer all emulsified. *Octyl alcohol, therefore, when thoroughly emulsified with serum, can introduce a dilution error up to about 2 per cent in analyses depending upon measurement of volume.* There is, of course, the possibility of an increased solubility of CO_2 in the octyl alcohol, but the coincidence of the calculated and determined errors indicates that such an error is insignificant. Under the condition of the pK′ determination reported below, with the constant proportion of serum and octyl alcohol, the error has been assumed to be constant at 1 per cent and all total CO_2 determinations corrected on that basis. The dilution error does not affect the pH measurement since that is a function of the ratio of $BHCO_3$-H_2CO_3 in the water phase.

With proper allowance for the dilution error, therefore, the Simms electrode vessel provides an exceptionally convenient method for saturating serum with CO_2 at known CO_2 tension, especially when a CO_2 mixture of known concentration is available.

Application of Above Technique to Indirect Determination of pH—Many workers have preferred to determine the pH of serum indirectly by constructing the CO_2 absorption curve, determining the CO_2 as drawn, and from these data estimating the CO_2 tension *in vivo*. The pH is then calculated, with a pK′ value for serum at 38° of 6.10, which is about the average of the determinations of Warburg, of Cullen, Keeler, and Robinson, and of Hastings, Sendroy, and Van Slyke (see Hastings, Sendroy, and Van Slyke (1928) for review and recalculation of values).

Eisenman (1926–27) extended the usefulness of this method by establishing the average slope of the CO_2 absorption curve and with it constructing the CO_2 absorption curve from one saturation with CO_2. She found that in forty human sera, including both normal and pathological, the differences in CO_2 content of separated serum for a change of 30 mm. of CO_2 tension (30 to 60 mm.) averaged 5.6 volumes per cent of CO_2, with variations from 2.9 to 9.0 but with 90 per cent of the values between 4 and 7 volumes per cent. For this range the slope $dCO_2/dpCO_2$ of the absorption curve varies between 0.13 and 0.23, with an average of 0.19. Eisenman suggests that this value may be constant for the slopes of the absorption curves of all mammalian sera. Although we feel that with such variations it is advisable to determine two or more points in constructing the absorption curve, it is interesting that the one human serum shown in Fig. 1 has an absorption curve with a slope of 0.21, which is close to Eisenman's average and the slopes of the absorption curve of six sera from four dogs average 0.167 with a spread of only 0.003. We also have evidence that the curve of separated rabbit serum has a similar slope.

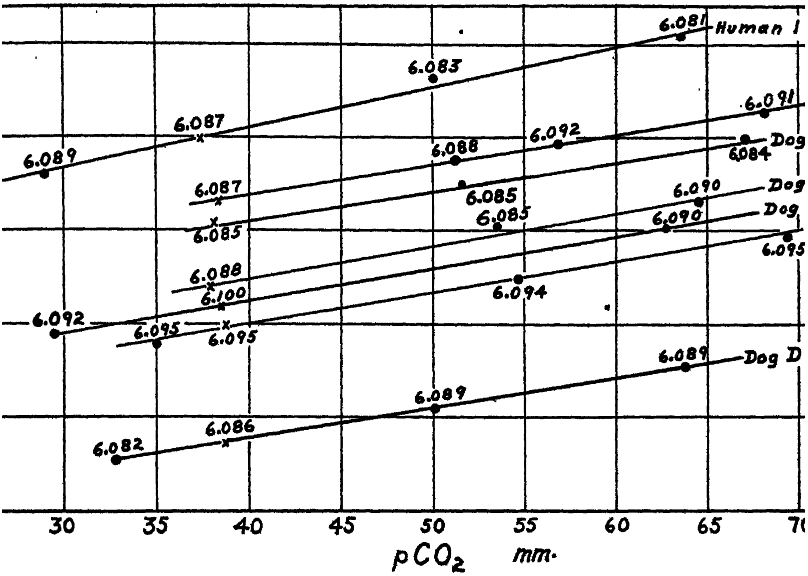


FIG. 1. Sera equilibrated in the Simms electrode at several varying CO_2 tensions. The numbers at the separate points are the pK′ values calculated for that determination. The points marked (×) are those made with the gas mixture from the cylinder containing 5.56 per cent CO_2 in hydrogen.

The Simms electrode vessel affords the most convenient method we know of for constructing the CO_2 absorption curve. Further, it also is available for estimating more directly the pH of serum as drawn from the CO_2 absorption curve, if this is plotted as total CO_2 against pH.

Application of Above Technique to Determination of Alkali

Reserve—In the work in which the Van Slyke apparatus was first used for studying acid-base balance of blood (Van Slyke and Cullen, 1917) a method for determining the alkali reserve of serum at a fixed standard CO_2 tension was suggested.

Although we now prefer for exact studies to determine the CO_2 content of serum as drawn, in the clinic it is still often most convenient, and sometimes most instructive to determine the alkali reserve. During the work reported here, it has become evident that the Simms electrode vessel, used with a cylinder of the CO_2 gas mixture of average alveolar air CO_2 content, affords a most convenient means of determining the alkali reserve at normal CO_2 tension. If the pH reading is taken at the same time, one has the pH at normal CO_2 tension. As a matter of fact, the pH can be read much easier than the CO_2 content can be determined and the alkali reserve can be easily calculated.

Details of Manipulation of Sera in the Simms Electrode—At the beginning of an experiment the heater and pump are started and the water bath is adjusted by additions of hot water to 38.3°. A thermometer calibrated to 0.1° is substituted for the electrode in the chamber. In about 10 minutes the system is in temperature equilibrium at 38°. The calomel half-cell is then transferred from the air bath to the system. The standard M/15 phosphate solution of $pH_{38} = 7.363$ is placed in the vessel, a freshly prepared platinum electrode inserted, and hydrogen bubbled through the solution at its constant rate for 15 minutes. A potential reading is made and the hydrogen allowed to flow for 10 minutes and another reading taken. These potential readings usually agree to within 0.1 millivolt of each other. (This procedure serves as control of the entire system and allows detection of abnormal or sluggish electrodes.) The systems having been found satisfactory against pure hydrogen, the gas inlet is switched to the hydrogen-CO_2 mixture which is allowed to flow for about 15 minutes to wash all pure hydrogen from the tubes and saline wash bottle. A potential reading checks the completeness of this washing. (When any given CO_2-H_2 mixture is to be used for a large series of determinations, the potential for the system is established for that mixture and the preliminary controls carried out directly with it.)

When the preliminary tests of the electrode system are satisfactory, the electrode is prepared for the test solution.

The phosphate solution is wasted, the electrode removed, and the chamber and electrode rinsed with distilled water. The chamber is dried with a clean towel and filter paper and a few drops of serum washed down the sides. The glass part of the electrode is dried with filter paper or cloth and the platinum plate rinsed with a few drops of serum.

3.6 cc. of serum and 0.04 cc. of octyl alcohol are put in the electrode vessel, the electrode inserted, and CO_2-H_2 bubbled through the serum for 15 minutes, the gas is stopped, and a potential reading made. The CO_2-H_2 is bubbled through for 10 more minutes and another reading made. Rarely, another 10 minutes are required for constancy of this reading.

After the serum in the Simms electrode has attained equilibrium, as shown by a constant potential, the hydrogen electrode is removed and the CO_2-H_2 mixture is allowed to bubble through the solution for a few minutes, while the manometric gas apparatus is made ready to receive the sample for CO_2 determination. When ready, the flow of gas is stopped and the Van Slyke-Ostwald pipette is inserted into the Simms vessel and the sample for the CO_2 determination is withdrawn at once before any change in the equilibrium can take place. Therefore, the CO_2 determination is made on the same solution on which the pH is determined.

Summary of Experiments with the Simms Electrode—The experiments reported above prove that the Simms electrode is entirely trustworthy for determining the pH of serum at any CO_2 tension. It is most convenient for saturating serum with CO_2. When octyl alcohol is used to prevent foaming, a correction of 1 per cent for dilution must be applied to the total CO_2 determination. The technique used in this laboratory is given in detail.

Results of pK′ Determination on Sera

Before discussing these results one question must be answered; *i.e.*, whether or not change in CO_2 tension itself affects pK′. In connection with the control experiments mentioned above, we determined on seven dog sera and one human serum the pK′ at several points of varying CO_2 tensions. These results are given in Fig. 1 and it can be seen at once that the pK′ is not affected by changes, within physiological limits, of pH or CO_2 tension.

These curves also illustrate the accuracy of the saturation of

TABLE I—*pK' of Normal Dog Sera Equilibrated at 38° with CO_2-H_2 Mixt*

peri- ent No.	Date	pCO_2	$[CO_2]$	$pH_{e_{38°}}$	pK'	Dog No.	Experiment No.	Date	*pC*
		mm.	*vol. %*						
1	5-23-32	38.7	44.0	7.295	6.091	C ♂	18	3-10-33	6
2	5-26-32	38.3	57.6	7.416	6.085		19	3-23-33	6
3	6-28-32	38.3	46.4	7.313	6.081		20	3-23-33	3
4	7-13-32	38.7	39.0	7.227	6.081		21	3-23-33	2
5	7-25-32	38.5	46.7	7.313	6.080		22	5-10-33	3
6	9-19-32	38.6	51.9	7.366	6.086		23	5-17-33	3
7	10- 6-32	38.5	49.2	7.337	6.080		24	5-24-33	3
8	12- 5-32	38.4	53.0	7.378	6.087		25	7-27-33	3
9	1-30-33	38.4	53.3	7.374	6.080		26	7-27-33	3
10	3- 2-33	38.7	55.5	7.392	6.083		27	7-27-33	3
11	5-11-33	38.4	49.5	7.350	6.090		28	7-29-33	3
12	5-22-33	38.5	54.9	7.395	6.088		29	10-10-33	3
13	12- 5-33	35.7	49.8	7.385	6.088		30	10-10-33	4
14	12-22-33	35.8	51.6	7.400	6.088		31	10-23-33	3
15	12-22-33	40.6	52.5	7.352	6.090		32	10-23-33	41
16	1-23-34	36.1	47.2	7.357	6.092		33	12- 6-33	3
17	1-23-34	41.0	48.1	7.308	6.091		34	12-18-33	36
1	6- 1-32	38.6	55.8	7.402	6.089		35	12-18-33	41
2	6- 6-32	38.3	44.5	7.297	6.084		36	1-19-34	36
3	6-16-32	38.3	50.8	7.358	6.085	D ♀	1	7-15-32	38
4	6-24-32	38.5	48.7	7.336	6.084		2	7-18-32	38
5	7-12-32	38.7	52.1	7.368	6.088		3	7-19-32	38
6	7-26-32	38.2	56.0	7.406	6.086		4	7-26-32	38
7	9-23-32	38.7	49.0	7.360	6.108		5	7-27-32	38
8	9-26-32	38.7	41.2	7.246	6.074		6	9-21-32	38
9	9-30-32	38.7	52.6	7.370	6.086		7	10- 3-32	38
10	10-14-32	38.8	54.3	7.381	6.083		8	10-24-32	38
11	10-28-32	38.9	52.4	7.370	6.089		9	12- 2-32	38
12	11-21-32	38.9	58.0	7.415	6.088		10	1-12-33	39
1	6-21-32	38.4	51.8	7.358	6.077		11	2-27-33	38
2	6-27-32	38.1	48.0	7.331	6.080		12	3- 7-33	37
3	7- 6-32	38.5	51.6	7.365	6.086		13	3- 7-33	38
4	7- 7-32	38.1	41.4	7.262	6.081		14	3-17-33	64
5	7- 8-32	38.4	49.1	7.340	6.082		15	3-17-33	50
6	7-12-32	38.7	53.7	7.384	6.091		16	3-17-33	38
7	7-27-32	38.3	50.8	7.363	6.090		17	3-17-33	32
8	9-20-32	38.4	47.4	7.324	6.082		18	4-24-33	67
9	9-27-32	38.5	55.2	7.404	6.094		19	4-24-33	51
10	10-17-32	37.9	54.4	7.397	6.087		20	4-24-33	38
11	11-10-32	38.1	49.8	7.355	6.089		21	5- 8-33	38
12	12-14-32	38.4	54.9	7.397	6.090		22	5-18-33	38
13	2-20-33	38.3	62.7	7.468	6.099		23	5-19-33	38.
14	3- 3-33	38.7	53.5	7.382	6.090		24	5-31-33	38.
15	3- 9-33	38.5	56.5	7.407	6.087		25	6-13-33	38.
16	3- 9-33	51.3	58.8	7.295	6.088		26	10- 2-33	36.
17	3-10-33	55.9	59.7	7.266	6.092		27	12-15-33	35

138 *Determinations on Sera of Eighteen Dogs*

O_2]	$pH_{e_{380}}$	pK′	Dog No.	Experiment No.	Date	pCO_2	[CO_2]	$pH_{e_{380}}$	pK′
%						*mm.*	*vol. %*		
.3	7.185	6.091	D ♀	28	12–15–33	40.5	49.1	7.326	6.094
.2	7.173	6.090		29	1– 4–34	35.8	40.7	7.285	6.084
.9	7.382	6.100		30	1– 4–34	40.7	41.4	7.248	6.097
.5	7.472	6.092		31	1–22–34	35.9	58.7	7.463	6.095
.1	7.339	6.083		32	1–22–34	40.6	59.6	7.410	6.090
.0	7.339	6.087	E ♀	1	9–28–32	38.6	61.8	7.446	6.087
.3	7.358	6.089	F ♂	1	10–31–32	38.4	54.4	7.381	6.078
.4	7.319	6.089		2	11–15–32	38.7	52.2	7.371	6.087
.6	7.269	6.089		3	3–15–33	69.4	54.7	7.126	6.095
.4	7.251	6.097		4	3–15–33	54.7	52.4	7.217	6.094
.2	7.405	6.097		5	3–15–33	38.8	49.9	7.355	6.095
.3	7.420	6.097		6	3–15–33	35.0	48.9	7.392	6.095
.4	7.373	6.098		7	5–15–33	38.5	57.5	7.421	6.095
.9	7.496	6.093		8	6–26–33	38.4	47.9	7.340	6.095
.1	7.465	6.097		9	7–31–33	38.6	38.4	7.232	6.091
.0	7.452	6.087		10	7–31–33	38.6	35.1	7.197	6.098
.2	7.404	6.103		11	7–31–33	38.5	33.4	7.177	6.102
.5	7.361	6.107		12	9–26–33	35.9	43.4	7.326	6.095
.5	7.409	6.095		13	10– 3–33	36.2	38.8	7.276	6.100
.7	7.321	6.088		14	10–18–33	36.2	66.7	7.529	6.107
.1	7.318	6.083		15	10–26–33	36.0	60.1	7.475	6.096
.2	7.376	6.085		16	12–19–33	36.1	39.3	7.285	6.102
.1	7.326	6.076		17	12–19–33	40.9	40.0	7.232	6.099
.2	7.301	6.081	G ♀	1	12–16–32	39.2	48.6	7.331	6.088
.3	7.378	6.091	H ♂	1	3– 1–33	38.5	49.4	7.339	6.080
.8	7.392	6.084		2	3–14–33	64.5	56.6	7.172	6.090
.3	7.352	6.085		3	3–14–33	53.5	55.3	7.243	6.085
.8	7.323	6.072		4	3–14–33	38.0	52.0	7.376	6.088
.0	7.400	6.091		5	5–12–33	38.4	49.9	7.358	6.095
.6	7.426	6.093		6	5–23–33	38.4	55.2	7.392	6.082
.3	7.446	6.086		7	6–19–33	38.6	50.2	7.350	6.086
.7	7.439	6.091		8	9–28–33	35.9	49.3	7.465	6.094
.8	7.089	6.089	J ♂	1	6– 9–32	38.4	50.6	7.360	6.088
.4	7.186	6.089		2	6–23–32	38.4	44.7	7.292	6.079
.5	7.285	6.086	K ♂	1	5–24–32	38.6	39.3	7.250	6.099
.7	7.347	6.082		2	6– 2–32	38.5	51.1	7.369	6.096
.9	7.173	6.084	L ♂	1	6– 8–32	38.3	47.4	7.327	6.085
.5	7.279	6.085	M ♀	1	7– 5–32	38.3	56.1	7.386	6.067
.4	7.400	6.085		2	7– 8–32	38.4	52.2	7.369	6.083
.4	7.300	6.075	N ♀	1	7– 9–32	38.6	39.2	7.230	6.080
.9	7.373	6.095	P ♂	1	7–11–32	38.7	53.4	7.382	6.089
.7	7.376	6.089	R ♂	1	11–23–32	38.6	55.0	7.395	6.089
.2	7.394	6.084	S ♂	1	11–28–32	39.3	52.3	7.368	6.094
.9	7.357	6.088	T ♂	1	1– 4–33	38.2	54.2	7.391	6.088
.0	7.396	6.106	U ♂	1	1–14–33	38.7	54.6	7.391	6.090
.1	7.378	6.097		2	2–10–33	39.1	56.7	7.410	6.097

serum in the Simms electrode in that they are in entire agreement with the many similar curves reported before.

With the technique detailed above, which we believe is reproducible to 0.005 pK′, we have determined the pK′ on 138 sera from eighteen normal dogs, on twenty-two determinations from fourteen dogs following various experimental conditions, and on 65 sera from twenty-two human subjects.

The results are condensed in Tables I to III. They are also

TABLE II

pK′ of Dog Sera following Experimental Procedures, Equilibrated at 38° with 5.56 Per Cent CO_2 in H_2 in the Simms Electrode

Dog No.	Date	Condition	pCO_2	$[CO_2]$	$pH_{e_{38°}}$	pK′
			mm.	*vol. %*		
V ♂	6–18–32	Parathormone intoxication	38.5	54.4	7.379	6.076
W ♀	6–18–32	NH_4Cl acidosis	38.4	36.8	7.194	6.071
L ♂	6–20–32	Diphtheria toxication	38.5	35.8	7.186	6.076
Y ♂	7– 1–32	Overdose irradiated ergosterol	37.8	58.1	7.424	6.084
J ♂	7– 7–32	$HgCl_2$ poisoning	38.2	33.9	7.172	6.084
P ♂	7–16–32	" "	38.3	27.3	7.057	6.074
M ♀	7–21–32	" "	38.4	30.7	7.118	6.079
R ♂	12– 6–32	Na barbital anesthesia	38.3	59.5	7.439	6.094
B ♀*	12– 8–32	Alkalosis by gastric juice removal, Na barbital anesthesia; histamine administration†	39.1	75.0	7.548	6.107
			39.0	79.6	7.572	6.104
H ♂	12–16–32	Ether anesthesia	39.2	53.3	7.374	6.089
A ♀	6– 3–32	Prepartum 36 hrs.	38.6	46.1	7.331	6.106
	6–10–32	Postpartum 5 days	38.4	44.6	7.303	6.091
F ♂	10–31–32	4 hrs. after acute hemorrhage	38.2	56.4	7.407	6.084
	11– 1–32	24 " " " "	38.8	56.9	7.399	6.079
	6–26–33	4 " " " "	38.3	47.7	7.339	6.094
	6–27–33	24 " " " "	38.5	48.6	7.374	6.096
D ♀	6–13–33	4 " " " "	38.6	47.2	7.318	6.082
	6–14–33	24 " " " "	38.8	50.5	7.355	6.090
	6–15–33	48 " " " "	38.9	56.5	7.397	6.083
H ♂	6–19–33	4 " " " "	38.6	51.8	7.357	6.078
	6–20–33	24 " " " "	38.6	50.1	7.352	6.089

* The first set of readings was obtained at 9.30 a.m.; the other at 2.40 p.m.

† Hypochloremia (aspiration of gastric juice).

TABLE III

pK' of Human Sera Equilibrated at 38° with CO_2-H_2 Mixtures in the Simms Electrode

Subject No.	Date	Condition	pCO_2	$[CO_2]$	$pH_{e_{38°}}$	pK'
			mm.	*vol.* %		
1 ♂	10–28–32	Normal	38.7	63.5	7.460	6.090
	3–20–33	"	37.6	59.9	7.444	6.087
	3–20–33	"	29.0	58.0	7.548	6.089
	3–20–33	"	50.1	63.2	7.334	6.083
	3–20–33	"	63.6	65.5	7.238	6.081
	3–29–33	"	38.9	63.6	7.470	6.101
	1–24–34	"	36.0	63.2	7.486	6.084
	1–24–34	"	40.8	64.4	7.449	6.095
2 ♂	11– 4–32	"	39.0	64.1	7.459	6.088
	3–31–33	"	38.2	67.0	7.485	6.084
3 ♂	3–28–33	"	38.8	66.4	7.480	6.090
4 ♂	3–30–33	"	38.5	65.8	7.472	6.083
5 ♂	10–12–32	Von Recklinghausen's disease	38.5	58.7	7.420	6.083
	10–20–32	" "	38.5	64.5	7.465	6.085
	1– 4–33	" "	38.4	62.1	7.442	6.077
6 ♀	11– 2–32	" "	38.9	59.4	7.423	6.085
	11–29–32	" "	39.1	60.0	7.423	6.083
20 ♂	6–12–33	" "	38.7	60.9	7.447	6.095
7 ♀	10–21–32	Fragilitas ossium	38.7	62.4	7.447	6.084
8 ♂	11– 8–32	Severe dental caries	38.2	62.1	7.442	6.077
28 ♂	11–22–33	Multiple sclerosis	40.6	63.2	7.460	6.113
	11–24–33	After 3 hrs. heat treatment	41.1	64.1	7.444	6.095
	12– 9–33	" 2¾ " " "	36.2	57.6	7.441	6.085
10 ♀	10–20–32	Cirrhosis of liver	38.5	52.3	7.382	6.097
	10–26–32	" " "	38.2	46.0	7.313	6.083
24 ♀	10–19–33	" " "	36.0	48.5	7.389	6.109
36 ♀	1–10–34	" " "	36.2	63.9	7.493	6.090
25 ♀	10–24–33	" " "	40.6	58.9	7.417	6.102
29 ♀	11–24–33	Toxic hepatitis	41.0	80.7	7.561	6.109
26 ♀	11– 8–33	Obstructive jaundice	36.1	55.9	7.449	6.105
	11–24–33	" "	41.0	76.2	7.540	6.113
27 ♂	11–10–33	" "	36.3	58.5	7.480	6.117
	12– 8–33	" "	35.9	42.6	7.332	6.109
31 ♀	12– 8–33	" "	35.8	65.7	7.523	6.102
17 ♂	10– 7–32	Renal rickets	38.7	33.8	7.170	6.091
15 ♂	9–28–32	Nephritis after scarlet fever	38.6	40.6	7.248	6.081
34 ♂	1–10–34	" " " "	36.3	53.1	7.415	6.096
16 ♀	4–28–33	Nephritis	38.3	62.7	7.459	6.090
18 ♂	6– 7–33	"	38.3	58.3	7.423	6.087

TABLE III—*Concluded*

Subject No.	Date	Condition	pCO_2	$[CO_2]$	$pH_{e_{38}°}$	pK′
			mm.	*vol.* %		
19 ♂	6- 8-33	Nephritis	38.3	73.1	7.527	6.089
	7-15-33	"	38.3	61.9	7.459	6.096
11 ♀	10- 7-32	Nephrosis	38.8	42.1	7.253	6.071
	10-18-32	"	38.2	43.9	7.290	6.082
	11-28-32	"	39.5	54.2	7.365	6.076
	1-23-33	"	38.3	47.0	7.319	6.081
	6- 1-33	"	38.7	57.1	7.412	6.089
12 ♂	11-18-32	"	38.5	52.3	7.373	6.090
	2-13-33	"	38.7	51.5	7.379	6.103
13 ♀	4-29-33	"	38.5	39.0	7.240	6.090
14 ♂	4-21-33	"	38.5	64.3	7.472	6.093
23 ♂	10- 6-33	"	35.8	47.6	7.374	6.099
	11- 8-33	"	36.2	49.5	7.395	6.108
	1-15-34	"	36.2	54.2	7.434	6.106
30 ♀	11-28-33	"	40.9	54.5	7.389	6.113
35 ♂	1- 8-34	Anemia	36.1	55.8	7.426	6.082
9 ♂	1-25-33	Scarlet fever	37.9	64.4	7.475	6.088
37 ♀	1-18-34	Septicemia	36.0	65.9	7.515	6.096
32 ♂	1- 6-34	? diagnosis	36.1	57.3	7.444	6.088
21-M ♀	7- 5-33	At childbirth	38.7	47.9	7.342	6.101
21-I		Blood, infant cord	38.7	56.0	7.404	6.091
21-M ♀	7- 6-33	24 hrs. after delivery	38.6	55.1	7.407	6.100
21-M ♀	7-13-33	8 days " "	38.4	55.4	7.408	6.097
22-M ♀	7-11-33	At childbirth	38.3	48.0	7.339	6.092
22-I		Blood, infant cord	38.3	53.5	7.381	6.084
22-M ♀	7-13-33	48 hrs. after delivery	38.3	59.3	7.439	6.095

analyzed statistically and a histogram has been constructed for comparison of our series with previous work (Fig. 2).

In the series of 138 determinations on apparently normal dog sera, we obtained a mean value for pK′ of 6.089 with a probable error of ±0.0004. The standard deviation for this series is ±0.0071. A pK′ variation of 0.01 would include 89 per cent of the determinations.

Study of various abnormal conditions has not revealed to us any consistent variation about the mean of our values. Some of the dogs have been studied steadily over a year, including the hottest summer weather, without any regular fluctuation. The

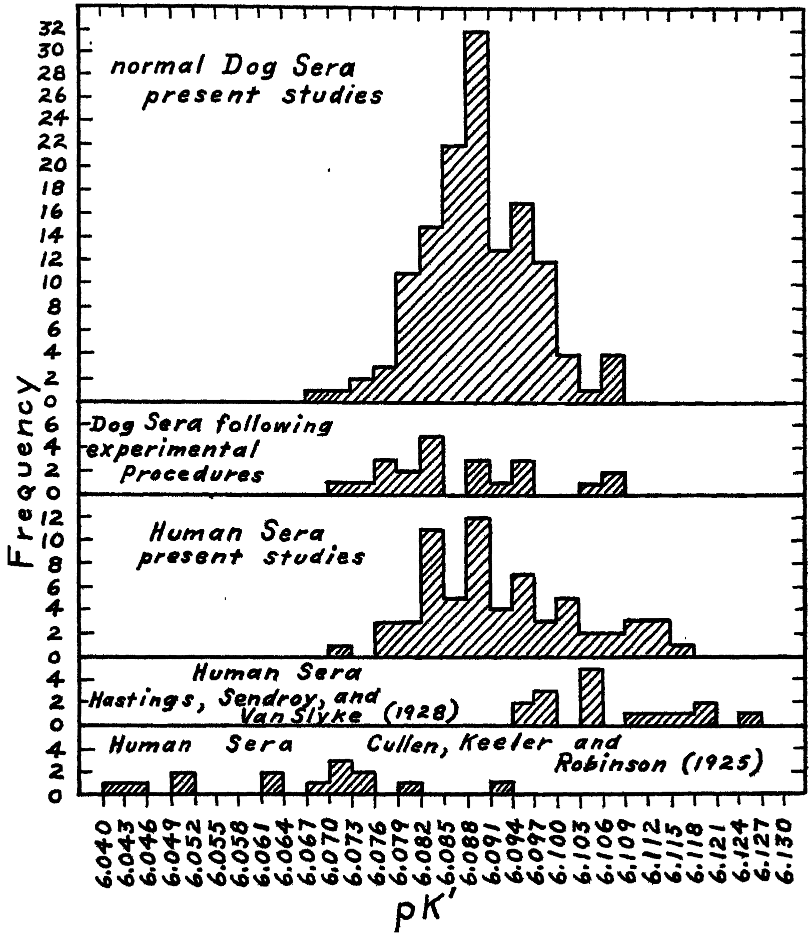


FIG. 2. Histogram for present and previously reported pK′ values for sera. The mathematical values are as follows:

	Mean pK′	Probable error of mean	Standard deviation	Probable error of standard deviation
138 dogs (normal)	6.089	±0.0004	±0.0071	±0.0003
22 " (experimental)	6.087	±0.0014	±0.010	±0.0010
65 humans	6.092	±0.0009	±0.0103	±0.0006
16 " (Hastings, Sendroy, and Van Slyke)	6.106	±0.0015	±0.0090	±0.0011
14 humans (Cullen, Keeler, and Robinson)	6.063	±0.0030	±0.0170	±0.0022

sera following experimental procedure on dogs was, with the exception of the hemorrhage studies, taken from dogs used by Dr. Guest and his colleagues for studies of the various procedures on the phosphate distribution in blood, the details of which will be reported elsewhere. In this series we have twenty-two determinations with a mean of 6.087 and a probable error of ±0.001. The distribution is the same as with the normals.

The number of normal human sera is not sufficient for a separate histogram, but since the range of the pK′ values of all our human material falls within that of the normal dogs, it seems justifiable to place all the human sera in one diagram. In the series of 65 determinations we obtained a mean value of 6.092 with a probable error of ±0.00086.

The pathological human sera likewise show no variation peculiar to individual pathology. In the nephrosis group, all had low total serum protein with reversed albumin-globulin ratio, but the pK′ values show no tendency toward the slight elevation suggested in the small nephritic group reported by Hastings, Sendroy, and Van Slyke. It is impossible to draw any conclusions as to variations of pK′ with a particular pathological condition. In the small series of cases in which the liver is involved, there is a suggestion that the pK′ value tended to be slightly increased; the average for eleven determinations on ten cases is 6.103.

It is evident that the pK′ of dog and human sera is practically identical. It is also evident from Fig. 2 that the values of the present series lie between those previously reported by Van Slyke, Hastings, and Sendroy, and by Cullen, Keeler, and Robinson.

Conclusions—The results of this study indicate that the system $BHCO_3/H_2CO_3$ in dog and human sera is practically identical; that the value for pK′ is surprisingly constant, with a standard deviation of less than 0.01 pK′; that it is not appreciably affected by pathological conditions; and that the pH may be calculated from a pK′ value of 6.09 with an expected accuracy, as far as the pK′ is concerned, of 0.01 pH.

SUMMARY

Technique, involving the use of the Simms bubbling electrode, is described for increased accuracy in the determination of pK′, for saturation of sera with known CO_2 tensions, and for determination of the alkali reserve.

The pK′ values have been determined on 138 sera from eighteen normal dogs, on twenty-two dog sera from fourteen dogs following various experimental procedures, and on 65 human sera from twenty-two subjects.

The pK′ values of human and dog sera are identical, averaging 6.09 with a standard deviation of ±0.008.

Various abnormal conditions do not appreciably influence pK′.

BIBLIOGRAPHY

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, **54**, 121 (1922).

Clark, W. M., *J. Biol. Chem.*, **23**, 475 (1915); The determination of hydrogen ions, Baltimore, 3rd edition (1928).

Cullen, G. E., *J. Biol. Chem.*, **52**, 521 (1922).

Cullen, G. E., Keeler, H. R., and Robinson, H. W., *J. Biol. Chem.*, **66**, 301 (1925).

Eisenman, A. J., *J. Biol. Chem.*, **71**, 611 (1926–27).

Hasselbalch, K. A., *Biochem. Z.*, **30**, 317 (1910).

Hastings, A. B., Murray, C. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **71**, 723 (1926–27).

Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924); **65**, 445 (1925).

Hastings, A. B., Sendroy, J., Jr., and Van Slyke, D. D., *J. Biol. Chem.*, **79**, 183 (1928).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, 1 (1931).

Robinson, H. W., *J. Biol. Chem.*, **82**, 775 (1929).

Schmidt, C. L. A., *J. Biol. Chem.*, **25**, 63 (1916).

Simms, H. S., *J. Am. Chem. Soc.*, **45**, 2503 (1923).

Sørensen, S. P. L., and Linderstrøm-Lang, K., *Compt.-rend. trav. Lab. Carlsberg*, **15**, 40 (1924).

Stadie, W. C., and Hawes, E. R., *J. Biol. Chem.*, **77**, 241 (1928, *a*); **77**, 265 (1928, *b*).

Stadie, W. C., O'Brien, H., and Laug, E. P., *J. Biol. Chem.*, **91**, 243 (1931).

Van Slyke, D. D., *J. Biol. Chem.*, **52**, 525 (1922).

Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **30**, 289 (1917).

Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **73**, 127 (1927).

Van Slyke, D. D., Sendroy, J., Jr., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, **78**, 765 (1928).

Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **95**, 547 (1932).

Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, **56**, 765 (1923).

Warburg, E. J., *Biochem. J.*, **16**, 153 (1922).

FURTHER INVESTIGATIONS CONCERNING THE NEW VITAMIN B GROWTH-PROMOTING FACTOR FOR RATS, FOUND IN WHOLE WHEAT*

By NELLIE HALLIDAY

With the Assistance of Linnea Dennett

(*From the Section of Home Economics, Michigan Agricultural Experiment Station, East Lansing*)

(Received for publication, March 28, 1934)

Since vitamin B_4 was first reported by Reader (1), a number of new factors for rat growth have been reported in this country and in England, and data were presented in some cases as to their physical, chemical, and biological characteristics. In the interest of brevity a review of the literature and discussion of the possible relationship of these factors to each other are omitted in this paper.

We have reported (2) the presence (and biological activity) of a factor found in whole wheat, which supplements vitamins B[1] and G in the diet of the rat. In the absence of this factor, growth ceases after about 6 or 8 weeks. If no additional supplement is given, the animals develop a nerve condition[2] very different from polyneuritis; the tendency of the animal is to roll around, in walking to lean to one side, and to sit hunched up leaning against the side of the cage. Swollen paws are sometimes encountered. Death occurs after about 3 months, following a very rapid loss of weight. Since these symptoms so closely resembled those reported by Reader, we believed that we were probably dealing with vitamin B_4.

---

* Published from Michigan Agricultural Experiment Station as Paper 179, new series.

Presented in part before the First annual meeting of the American Institute of Nutrition at New York, March, 1934.

[1] In this paper the terms vitamin B and vitamin G are used to designate the two factors commonly known as vitamin B_1 and vitamin B_2, or as the antineuritic and growth-promoting factors, or as the heat-labile and heat-stable members of the vitamin B complex, respectively.

[2] We are indebted to Mr. B. B. Roseboom and Mr. R. V. Brown of the Physiology Department of Michigan State College for the following description of the deficiency. "Symptoms: Animals emaciated, general condition poor. Habitus, left half of body slightly lower than right. Muscle tone generally diminished, more marked on the left side. Left fore limb moderately adducted. Gait slightly ataxic; coordination slightly faulty; animal could stand on hind limbs and maintain its position with ease; tendency to move in circles toward the affected side. Possible ophthalmic involvement, but so slight as to make accurate judgment

It was further shown that the feeding of a small portion of whole wheat containing an almost negligible amount of vitamin B, in tests for the vitamin B content of our materials, would prevent the symptoms and induce a nearly constant growth rate throughout the experimental period. Such a method of testing, however, has the disadvantages of being laborious and of lessening the accuracy of the method, since a small amount of vitamin B is furnished. It was deemed advisable, therefore, to prepare, if possible, a concentrate of this factor which would contain little or no vitamin B. The use of such a concentrate would improve the tests for vitamin B, and the concentrate itself could be used for further studies of the new factor.

EXPERIMENTAL

In carrying out the experimental work several difficulties presented themselves. Whole wheat contained so large an amount of starch that extractions with dilute acid or alcohol were difficult. Secondly, it was necessary to standardize a method for determining this new factor. Thirdly, we had considerable difficulty with coprophagy, even though a $\frac{3}{8}$ inch or $\frac{1}{2}$ inch wire mesh was used in the floor of the cage and all cages were raised at least 2 inches above the pan.

---

impossible; no evidence of nystagmus. These symptoms seem to indicate a possible involvement of the vestibular nuclei or of the vestibular branch of the VIII nerve."

In addition two of our deficient animals were sent to Dr. Charles F. Church of the University of Pennsylvania for examination. Dr. Church writes: "These rats show in a very marked degree the symptoms which I have found characteristic of deficiency in the heat-labile factors of yeast... It is evident from the similarity of symptoms that we are working with the deficiency of similar, if not identical, factors. The increased duration of the vestibular test, which I found to be characteristic of animals deficient in the heat-labile fraction of yeast, is also present in your two animals."

The problem of coprophagy appears to be solved at present by the use of the anticoprophagy harness described in detail by Page (3). Contrary to the finding of Booher and Kaneko (4) we find that the accuracy of the tests for both vitamins B and G is improved to almost the same extent by this method. The growth rate is lessened, with the same supplement, in a harnessed animal, and it seems probable that the unharnessed rat supplemented its diet with feces. It was necessary, after starting this technique, to restandardize certain materials, but all of the results given are for harnessed animals.

Reader (5) described a method of testing for her factor by the use of adult animals. This method has the advantage that an animal may be used for successive tests, and thus act as a check on itself. We used this method in our first reported work and to some extent have continued to do so. However, in order to obtain quantitative results so as to compare the potencies of various materials, we find that a slightly different technique is advantageous.

Protein-free milk has been shown to be a good source of vitamin B and to contain relatively little of the new factor (2). Animals receiving this material grow for about 6 weeks, after which the growth curve flattens and nerve symptoms begin to be manifested. At that point, if a supplement is given which contains the new factor, growth is resumed, and it is roughly proportional to the amount of the supplementary portion. A litter mate which receives the protein-free milk only will remain almost constant in weight. Thirteen such animals gained on an average 1 gm. during the 4 week period after the initial growth had ceased. If the weekly growth rate of these animals is used as the base-line, and the growth of litter mates receiving an additional supplement is plotted as gain above such control, a fair test of the potency of the supplement—as regards the new factor—appears to be possible. We have, therefore, used this method, but with frequent recourse to the "adult" method in order that we might have checks on the same animals. The results shown in Fig. 2 are derived in this manner.

The basal diet used is that of Chase and Sherman (6) for the determination of vitamin B. This diet carries 15 per cent of autoclaved yeast as a source of vitamin G. In many laboratories the custom of feeding all vitamin supplements separately from the basal diet has been followed. Chick[3] has stated that some of the symptoms ascribed to vitamin B_4 deficiency, and to the deficiency we reported, might be due to a submaximal allowance of vitamin G. We have, however, tested each lot of autoclaved yeast, and have kept careful records of the intake of basal diet of all experimental animals. We feel confident, therefore, that, even when moribund, our animals were receiving an adequate allowance of vitamin G from the diet. It must be borne in mind also that they were all receiving protein-free milk, which we have shown (7) to be a good source of vitamin G.

Harris[4] and coworkers criticized our technique as supplying too low an allowance of vitamin B. They believe that the nerve condition is the result of a chronic vitamin B deficiency. Though it is true that our protein-free milk carries some of the new factor and, therefore, cannot be fed *ad libitum,* we believe that certain of the results obtained, which will be described later, will answer this question.

Since whole wheat was found to be a rather unsatisfactory material with which to proceed, we had the wheat milled and tested the various fractions; bran, middlings (Fraction 1, the outer portion; Fraction 2, carrying the larger part of the germ), and also a sample of commercial wheat germ. The wheat germ proved the richest source of both factors. Bran was lower than the germ in vitamin B potency, but, since it contained nearly as much of the new factor and was almost devoid of fat, we used it for further work. It appears to be more constant than yeast in vitamin B potency. Three different lots of yeast when fed at a level of 1.8 gm. weekly for vitamin B gave widely different results (Fig. 1).

During the course of these experiments the English workers (8) reported the preparation of a crystalline concentrate of vitamin B_4 from yeast. Since we had stated that we thought we were dealing with this vitamin, we attempted to prepare such a concentrate from wheat as the best method of settling this point.

It was necessary to prepare an extract with which to work, and for this we tried ether, dilute alcohol, and dilute acid.

---

[3] Chick, H., personal communication.

[4] Harris, L. J., personal communication.

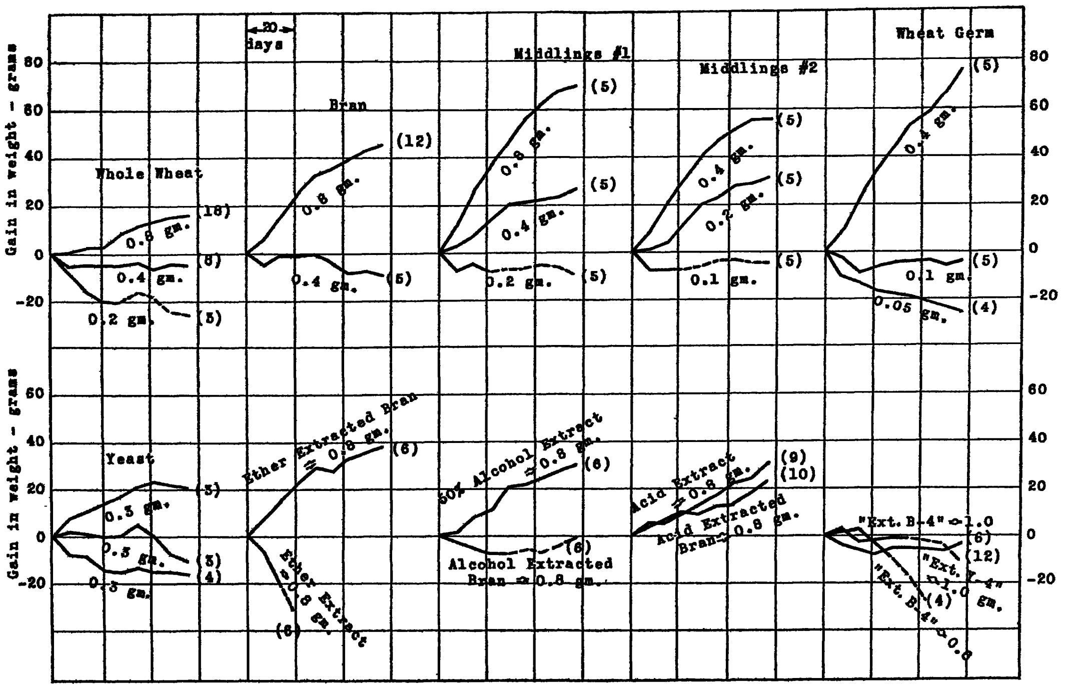


Fig. 1. Average growth curves of rats which received the Chase and Sherman vitamin B-deficient diet plus graded portions of the materials indicated. The figure in parentheses at the end of each curve indicates the number of animals for which results are included. The broken line indicates that one or more rats have died, and the curve from this date represents results for the surviving animals.

Continuous extraction with ether for 6 hours was carried out in the hope that an extract might be prepared low in vitamin B and rich in the new factor. Under our conditions, however, ether removed very little of either factor, although Reader had reported that vitamin B_4 could be extracted by this means.

Extraction with 50 per cent alcohol removed more of the new factor (as determined by tests of the extracted bran), but the resulting extract contained a very gummy material, after removal of the alcohol. This proved difficult to handle quantitatively.

We finally used an aqueous extract. This was prepared by shaking bran for 2 hours with acidified water at about pH 4 (determined colorimetrically) in the proportion of 1 kilo of bran to 8 liters of water. It was prepared fresh at frequent intervals and kept in the cold for feeding. This extraction does not effect a separation of the two factors, but it provides a material which is easily prepared, readily eaten by the animals, and which carries about half of the original activity of the bran, as regards the new factor. It was from this extract that our concentrate was prepared.

A detailed description of the method of preparing the crystals is given by the English workers. We followed their method very carefully, with three rather important exceptions. Our bran extract was prepared as described above, whereas they used a hot aqueous extraction. We used lots of 1 kilo of bran, whereas they used 50 or 100 kilo lots of yeast; therefore, we used proportionately smaller amounts of reagents. In their work they found that vitamin B_4 was adsorbed on norit at pH 1 and vitamin B remained unadsorbed. In our work with bran (and to a lesser extent with yeast) we found that the greater part of the vitamin B had been adsorbed, and the filtrate after adsorption still contained about half of our new factor. Moreover, by elution with 50 per cent acid-alcohol we were only able to recover about 10 per cent of the original potency. It was necessary, therefore, to proceed with the subsequent steps of the crystallization process with this filtrate in the place of the alcoholic extract of the charcoal.

We feel that if other workers are interested in repeating our work it should be possible for them to do so, provided they observe the precautions mentioned by the English workers and substitute our methods at these three points. It appears to be necessary to test aliquots for both vitamins as often as is biologically possible, so as to follow the path of the activity.

The technique used with animals is changed only slightly from that described in former papers. Standard rats at 26 to 28 days of age are subjected to a preliminary depletion period of about 2 weeks, during which time they receive the basal diet only. They are then put into harness, and given a supplement, care being taken to balance the various groups as to sex and weight. All portions are given on a 6 day week basis, and all extracts or residues are calculated to equivalents in unextracted material. Animals used in tests for the new factor are harnessed and fed protein-free milk until the litter as a whole is constant in weight. The second supplement is then added. Here the groups are balanced as carefully as possible as to sex, weight, and gain during the protein-free milk feeding period. One animal from each litter receives the protein-free milk supplement only, to act as a control.

Results

In Figs. 1 and 2 are shown average growth curves of the rats used in tests for the potencies of the materials. As is seen in Fig. 2, the method used for testing for our new factor is apparently fairly accurate, and reproducible results are obtainable. The ether extract shows no activity, and the extracted bran fed at two levels induced the same rate of gain as the unextracted material. The potency is equally divided between the acid extract and the extracted bran, and, within the limits of biological work, the potency appears to be quantitatively accounted for.

The materials designated as Extract Y-4 and Extract B-4 are the filtrates remaining after the adsorption on norit. It seems apparent that most of the vitamin B is adsorbed, and fully half of the new factor is left in the filtrate.

The subsequent steps of the crystallization process resulted in materials which it was difficult or impractical to test biologically. We attempted to feed the filtrate remaining after precipitation of the phosphotungstate, but in no case would the animals consume a sufficient amount to give accurate results.

Reader and coworkers had tested the final acetone solution as a check on their material before crystallization, and we attempted to do so. However, owing to the smaller scale of preparation and

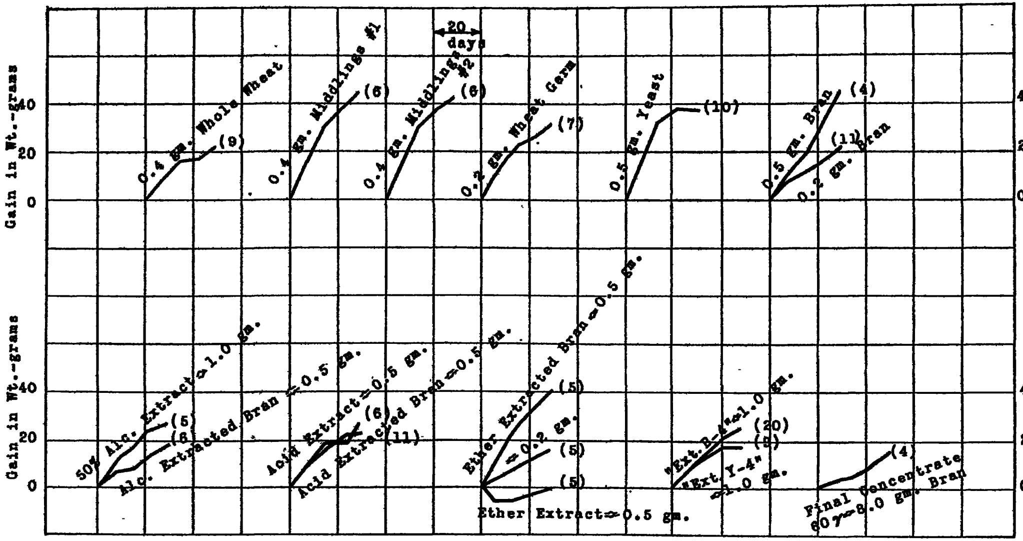


FIG. 2. Average curves showing growth of rats receiving protein-free milk plus the additional supplement indicated *above* the growth of litter mate controls which received protein-free milk only. All animals received the Chase and Sherman vitamin B-deficient diet. The figure in parentheses at the end of each curve indicates the number of animals for which results are included.

apparently to greater losses, it was necessary to feed larger amounts than could be tolerated by the animals.

The final product was obtained in yields of about 1 to 1.5 mg. from 1 kilo of bran. This was tested in daily portions of about 60 micrograms, representing approximately 8 gm. of bran. As is seen in Fig. 2, the growth curve resembles that obtained from 0.2 gm. of untreated bran. In view of the very small yields, we did not feed larger amounts, nor did we test the crystals for their vitamin B content.

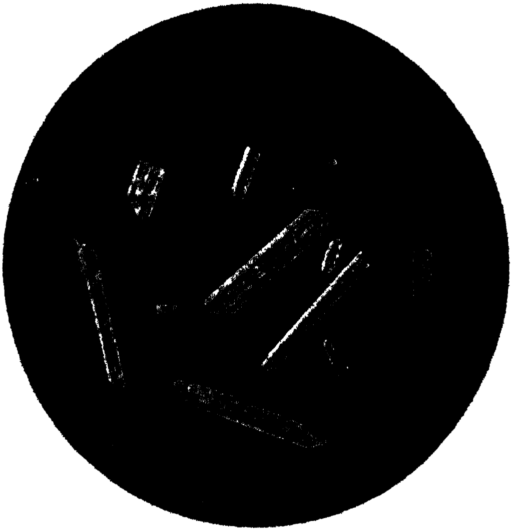


FIG. 3. Photomicrograph of the crystalline product (about 10 ×)

In biological testing two entire lots were used without drying or recrystallization. The material was dissolved in water and made up to a suitable volume for feeding. The crystals themselves were fed when sufficient material was available.

The author has conferred with Professor Peters and his staff, and the crystals were compared. Microscopically they appeared to be very similar (Fig. 3). The vitamin B_4-deficient rats closely resembled our deficient animals. Professor Peters kindly offered to test a sample of our crystals for the activity of vitamins B and B_4. This particular sample, however, had not been recrystallized. It was found to contain a variable amount of inorganic material, and it was not active in either factor in the amounts tested (40 micrograms for vitamin B_4 and 200 micrograms for vitamin B). It is unfortunate that a purified sample was not available and that there was not sufficient material for more complete tests.

TABLE I

Comparison of New Factor in Whole Wheat with Vitamin B_4

	Whole wheat factor	Vitamin B_4
Extracted with water	Yes	Yes
" " dilute acid	"	"
" " 50% alcohol	"	"
" " ether	No	" (cannot be recovered from the ether extract)
Adsorption on norit at pH 1 from aqueous extract	About 50% unadsorbed; balance not well recovered from norit	Yes
Precipitation of phosphotungstate between pH 3 and pH 1	Yes	"
Solubility of phosphotungstate in:		
Hot 50% alcohol	"	"
Cold 50% "	No	No
50% acetone	Yes	Yes
Positive reaction for pentose after acid hydrolysis	"	"
Crystalline product	"	"
M.p. of hydrochloride, °*C.*	Decomposes at 242	Sublimes at 220; decomposes at 248
" " picrate derivative, °*C.*	273	278

For vitamin B_4, the "adult" method was used. Cure of nerve symptoms and resumption of growth were taken as the criterion.

For testing the whole wheat factor, the vitamin B-deficient diet plus protein-free milk was fed until growth ceased. Growth induced in the test animals receiving supplementary feeding, above that of litter mate controls receiving protein-free milk only, was taken as the criterion.

DISCUSSION

As a result of the experiments described we cannot say that we are dealing with vitamin B_4. It seems evident that our factor is necessary for growth and well being of the rat; that the condition resulting from a deficiency of this factor in the diet of the rat closely resembles vitamin B_4 deficiency; that in certain physical and chemical characteristics our material is closely similar to vitamin B_4, but that there are numerous exceptions; and, finally, it seems hardly possible that the condition induced can be attributed to a deficiency of either vitamin B or G. Table I summarizes the data which we have.

It seems possible that the differences found between vitamin B_4, as isolated and described, and our product may be due to the fact that one or the other of the two products may be associated with impurities or contaminating substances.

Keenan and coworkers (9) reported that vacuum-dried hog liver is a good source of vitamin B_4. Preliminary tests of vacuum-dried hog liver in our laboratory show it to contain our factor, but it also contains a relatively high amount of vitamin B. Results from tests of various food materials will be presented in a later paper.

SUMMARY

1. Further studies have been made of the new vitamin factor found in whole wheat, and previously reported from this laboratory. Fractionation of the wheat showed the germ to be the richest source, but the bran proved a satisfactory material from which to prepare an extract.

2. The factor may be extracted from bran by dilute acid or dilute alcohol, but not by ether.

3. Following in the main the method described by Peters and coworkers, it has been possible to prepare a crystalline concentrate of the vitamin factor. Although in many respects there is close similarity between this factor and vitamin B_4, the differences are too great at present to warrant definite conclusions.

The author wishes to thank Elaine Come and Goldie Lieberman for assistance in the technical work.

BIBLIOGRAPHY

1. Reader, V., *J. Soc. Chem. Ind.*, **47**, 1247 (1928); *Biochem. J.*, **23**, 689 (1929).
2. Halliday, N., *J. Biol. Chem.*, **96**, 479 (1932); **98**, 707 (1932).
3. Page, J. W., *Proc. Soc. Exp. Biol. and Med.*, **30**, 87 (1932).
4. Booher, L. E., and Kaneko, T., *Proc. Soc. Exp. Biol. and Med.*, **30**, 69 (1932).
5. Reader, V., *Biochem. J.*, **24**, 1827 (1930).
6. Chase, E. F., and Sherman, H. C., *J. Am. Chem. Soc.*, **53**, 3506 (1931).
7. Halliday, N., *J. Biol. Chem.*, **95**, 371 (1932).
8. Barnes, H., O'Brien, J. R. P., and Reader, V., *Biochem. J.*, **26**, 2035 (1932); *J. Physiol.*, **76**, 8 P (1932). Kinnersley, H. W., Peters, R. A., O'Brien, J. R. P., and Reader, V., *Biochem. J.*, **27**, 225 (1933). Heard, R. D., Kinnersley, H. W., O'Brien, J. R. P., Peters, R. A., and Reader, V., *Nature*, **131**, 617 (1933).
9. Keenan, J. A., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, **103**, 671 (1933).

A STUDY OF TISSUE RESPIRATION AND CERTAIN REDUCING SUBSTANCES IN CHRONIC FLUOROSIS AND SCURVY IN THE GUINEA PIG

BY PAUL H. PHILLIPS, F. J. STARE, AND C. A. ELVEHJEM

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*)

(Received for publication, May 7, 1934)

Attention has been called to the marked similarity of the symptoms of fluorosis and scurvy in the guinea pig (17). A suggestion was made that chronic fluorine poisoning interfered in some manner with ascorbic acid or its function in the animal body. Studies on the distribution of a reducing substance (vitamin C) in the tissues of fluorine-fed cows as well as respiration of the suprarenal cortex indicated that ascorbic acid or vitamin C and respiration were disturbed in chronic fluorine poisoning (19). It is known that cattle synthesize vitamin C. The vitamin C content in the suprarenal gland and anterior lobe of the hypophysis increases in the fluorine-poisoned rat (18), another species known to synthesize vitamin C. These observations suggested that the mechanism of fluorine toxicosis and the mode of action of vitamin C could be profitably studied further in a species susceptible to scurvy.

Szent-Györgyi (24) in 1928 showed that hexuronic acid, now called ascorbic acid (vitamin C), played a rôle in certain biological oxidation-reduction mechanisms and suggested that vitamin C might have a similar function in the animal body (25). Because ascorbic acid reduced hemin to a green chromagen in air, von Euler and Malm (4) concluded that it functioned as a catalyst in oxidation-reduction enzyme systems. The evidence of Purr (20) and of Karrer and Zehender (11) suggests that ascorbic acid is capable of activating intracellular proteinases and in this respect it resembles glutathione and cysteine. Purr suggested that protein decomposition was dependent upon the presence of vitamin C,

---

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

and that the interaction of glutathione and ascorbic acid regulates the intracellular protein metabolism.

The distribution of ascorbic acid has been studied by various laboratories. Birch, Harris, and Ray (3), Bessey and King (1), Svirbely (23), and others have shown by the use of chemical methods that ascorbic acid is widely distributed throughout the body, and that it is highest in the more actively metabolizing tissues. The suprarenal cortex has long been known to be relatively rich in ascorbic acid. More recently Harris and Ray (6), and Huszak (10) have shown that the medulla of the suprarenal gland is likewise rich in ascorbic acid.

The distribution of glutathione in scorbutic animals has not attracted much attention. Sato and Ohata (21) concluded from a few scorbutic guinea pigs that glutathione was increased in the adrenal gland (about 43 per cent) over a like number of controls. Since the number of animals was few and the controls lost considerable weight during the experiment, an accurate interpretation of the data is difficult. Wolff and Manjean (27) reported glutathione values for a few organs from normal guinea pigs.

The importance of glutathione in cellular respiration has been shown by Hopkins and Elliott (9). Birch and Dann (2) studied the distribution of ascorbic acid and glutathione in animal tissues and suggested that ascorbic acid and glutathione might be two linked factors in one system of oxidation in the animal cell.

There is a paucity of data on the effect of scurvy upon tissue respiration. Söderström and Törnblom (22) measured the oxygen consumption of normal and scorbutic guinea pigs under basal conditions and found a reduction of O_2 consumption per minute per unit of body surface but concluded that their animals were too few in number to warrant the conclusion that the reduced O_2 consumption was due to vitamin C deficiency. Contrary to these results Mosonyi and Rigó (16) found the gaseous metabolism of scorbutic guinea pigs increased above normal. The administration of ascorbic acid reduced the gaseous metabolism in scorbutic guinea pigs and increased it in normal animals. Harrison (7) found that the rate of oxygen uptake, measured in an atmosphere of oxygen, of slices of liver from scorbutic guinea pigs was less than that of liver slices from normal animals, and that the *in vitro* addition of ascorbic acid increased the rate of oxygen uptake of the scorbutic liver but did not increase the uptake of the normal liver. On the other hand it is known that NaF inhibits cellular and tissue glycolysis.

The hypertrophy of the suprarenal gland during fluorine toxicosis and scurvy (17, 18) indicates that the vital processes of the gland itself may be affected. Hartman (8) and Lockwood and Hartman (15) have called attention to the supplementary effect of cortin to diets low in vitamins C and B_1. Thus a summary of the literature indicates that ascorbic acid (vitamin C) as well as glutathione may be concerned with tissue respiration and because of their oxidation-reduction properties function as proteolytic enzyme activators, that both substances have a systemic distribution which is most pronounced in the vitally active organs, and that little or no data are available on the influence of fluorosis upon these substances and their respective physiological functions.

The aim of this study was to determine the effect of fluorosis and of scurvy upon the distribution of the 2,6-dichlorophenolindophenol and the iodine-titratable substances (ascorbic acid) (glutathione), and upon the tissue respiration of several tissues from guinea pigs.

EXPERIMENTAL

Guinea pigs varying in weight from 140 to 350 gm. were divided into seven lots. The first three lots had ten animals each, while Lots IV to VI inclusive contained five animals each and Lot VII contained six animals. The basal ration used was the diet formerly used in this laboratory and consisted of rolled oats 69 parts, autoclaved alfalfa hay 25 parts, casein 5 parts, and 1 part NaCl.

The animals were fed the basal diet and 1 cc. of orange juice for 4 days preliminary to starting the experiment proper, in order to accustom them to handling and to their quarters. They were then placed upon the following regimen.

Lot I. Basal ration only (negative controls)
" II. " " + 3 cc. orange juice daily
" III. Diet of Lot II + F (25 mg. per kilo of body weight)
" IV. Basal ration + 6 cc. orange juice daily
" V. Diet of Lot IV + F (25 mg. per kilo of body weight)
" VI. Basal ration + 12 cc. orange juice + 25 mg. F per kilo of body weight
" VII. Basal ration + pure ascorbic acid (1 to 2 mg. daily for 25 to 28 days)

The fluorine was fed as a NaF solution by pipette daily to assure a constant fluorine intake. The orange juice was fed by pipette. The latter was fed between 8 and 9 o'clock in the morning followed by the NaF 4 to 6 hours later. The ascorbic acid was weighed out in daily doses, dissolved in water, and fed by pipette.

The animals were removed from the experiment when positive symptoms of scurvy, or fluorosis, had developed. As near as possible, positive control animals were removed the same day in order to provide normal tissues. The animals were killed by a blow on the head and thoroughly bled by severing the arteries and veins of the neck. The following organs were quickly removed and weighed *in toto* or portions thereof depending upon the size of the organ: the anterior lobe of the hypophysis cerebri, suprarenal glands, the liver, pancreas, kidney, and heart. One suprarenal gland, a portion of the liver, one kidney, and the pancreas were ground with acid-extracted sand and extracted repeatedly with 8 per cent trichloroacetic acid until 25 cc. of extract were obtained. Aliquot portions were then titrated with 2,6-dichlorophenolindophenol and 0.01 N iodine solution. The 2,6-dichlorophenolindophenol titration for ascorbic acid was made after the method of Bessey and King (1). The ascorbic acid titration was computed as vitamin C and converted to cc. of 0.01 N iodine equivalent. This was deducted from the iodine titration and the remainder of the iodine was calculated as glutathione.

The other suprarenal gland and sufficient liver were used for oxygen uptake studies in the Barcroft apparatus. The oxygen uptake was studied at 37° in plain phosphate-Ringer's solution buffer, pH 7.3, and also in buffer containing as substrates glucose, lactate, and succinate to a concentration of 0.2 per cent. The effect of added cyanide on these measurements was observed. The oxidase activity was qualitatively measured with Nadi's reagent following the method used by Keilin (12). Cytochrome was observed with a microspectroscope. The dehydrase activity of the liver was determined by Thunberg's technique (26).

Results

Fluorosis in guinea pigs produced a syndrome similar in many respects to the scorbutic syndrome. When 25 to 30 mg. of F were administered daily per kilo of body weight to guinea pigs on a diet adequate in vitamin C, a characteristic growth curve was obtained (Chart I); growth continued for some weeks and then declined rather sharply in most cases to death or to a very low plane of existence. The onset of symptoms was accompanied by lethargy, and "ruffing" of the fur, which produced a characteristic effect about the jaws and neck. As the poisoning became more pronounced lameness developed together with swollen joints, which was most pronounced in the knee joint. In severe cases the typical "face ache" posture was assumed when the animals were not disturbed. These symptoms were accompanied by anorexia and extreme emaciation. In this condition diarrhea was often present, a condition also noticeable in scurvy. Postmortem examination not infrequently showed intestinal irritation and hemorrhages, when acute symptoms and rapid loss of weight occurred. In all cases the syndrome was accompanied by a hypertrophy of the suprarenal glands, a fact also observed in the rat. One important difference between the syndrome of fluorosis and scurvy was the infrequent occurrence or absence of subcutaneous and intermuscular hemorrhages, which is usually taken as a distinctive characteristic of scurvy.

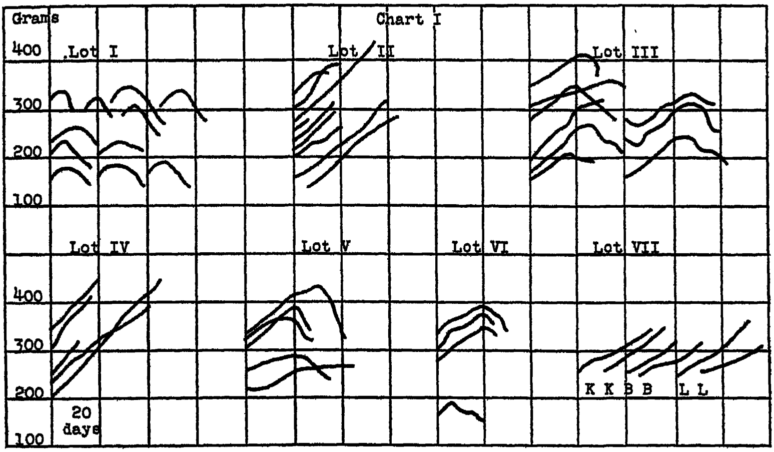


CHART I. Growth curves of guinea pigs fed various rations, the constituents of which are listed in the text for each lot. For Lot VII, the designations, K, B, and L represent curves obtained by Karrer, Bauman, and Link preparations, respectively.

The influence of scurvy and fluorosis upon the distribution of ascorbic acid and glutathione is shown in Table I. An inspection of the data in Table I shows that scurvy produces a decline in the amount of ascorbic acid present per unit of weight in all the tissues studied. A decrease of approximately 50 per cent occurred in the liver, kidney, and pancreas. Practically complete disappearance occurred in the anterior lobe of the hypophysis. Because of the smallness of the gland, the material available for analysis in this tissue makes the error admittedly large. However, a consistent reduction in the ascorbic acid content of the gland was obtained in the animals fed fluorine. In view of the vital hormonal relationships associated with this gland the importance of this result should not be overlooked. Fluorine did not apparently influence the ascorbic acid content of the other tissues. The ingestion of increased quantities of ascorbic acid, or vitamin C, causes a corresponding rise in the ascorbic acid content in the tissues studied, regardless of fluorine ingestion.

TABLE I

Influence of Fluorosis and Scurvy upon Reducing Substances in Tissues of Guinea Pigs

	Lot I. Basal ration (scorbutic)	Lot II. Basal + 3 cc. orange juice daily	Lot III. Basal + 3 cc. orange juice + NaF daily	Lot IV. Basal + 6 cc. orange juice daily	Lot V. Lot IV diet + NaF	Lot VI. Basal + 12 cc. orange juice + NaF
Average vitamin C values expressed in mg. per gm. fresh tissue						
Liver	0.039	0.074	0.074	0.155	0.161	0.232
Kidney	0.024	0.051	0.041	0.079	0.064	0.115
Pancreas	0.033	0.066	0.089	0.139	0.134	0.259
Hypophysis (anterior lobe)	Trace	0.544	0.491	0.747	0.300	0.518
Mg. glutathione calculated from iodine titration and expressed in mg. per gm. fresh tissue						
Liver	6.73	5.73	6.76	5.01	5.50	6.72
Kidney	3.68	3.79	3.67	3.72	3.69	3.52
Pancreas	3.03	2.83	3.08	2.84	2.76	7.89

Scurvy and fluorine toxicosis were found to be alike in that they caused an increase in the amount of reducing substance, or substances reacting with iodine (presumably glutathione) in the liver. The extent of the increase was approximately 18 per cent at ordinary levels of vitamin C feeding, and about 10 per cent when the vitamin C feeding was doubled. No appreciable change was apparent in the iodine-titratable substances in the kidney or pancreas, although the latter seemed to be slightly increased in the fluorine-fed animals.

TABLE II

Ascorbic Acid (Vitamin C) Found in Suprarenal Gland. Data Averaged by Lots

	Weight of suprarenal gland		Ascorbic acid per gm. fresh tissue	Total ascorbic acid present in suprarenal	
	Single gland	Both glands		Single gland	Both glands
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Lot I. Basal ration (scorbutic)............	102.7	169.8	0.150	0.015	0.025
Lot II. Basal + 3 cc. orange juice..........	92.1	157.7	0.474	0.044	0.075
Lot III. Lot II diet + NaF..................	96.0	202.9	0.277	0.027	0.056
Lot IV. Basal + 6 cc. orange juice..........	87.5	180.0	0.476	0.042	0.086
Lot V. Basal + 6 cc. orange juice + NaF...	113.0	240.0	0.528	0.060	0.127
Lot VI. Basal + 12 cc. orange juice + NaF...	110.6		1.012	0.112	
Lot VII. Basal + crystalline ascorbic acid...	67.4	138.6	0.25	0.017	0.035

Since ascorbic acid has been intimately associated with the suprarenal gland, the data obtained on the distribution of ascorbic acid in this gland in chronic fluorine toxicosis and scurvy have been compiled in Table II. It is seen that the ascorbic acid content of the suprarenal gland was relatively and absolutely decreased in scurvy. The first animals removed from the experiment were in the early stages of scurvy, or fluorosis, as the case happened to be. Withholding the first two animals removed from the experiment in Lot I from the average used to show the mg. of ascorbic acid per gm. of fresh tissue reduces the value from 0.15 mg. to 0.10 mg. of ascorbic acid per gm. of tissue. Similarly animals fed the fluorine exhibited various degrees of severity of fluorotic symptoms when sacrificed. While not entirely uniform and of the same grade of fluorine toxicosis, no animal was assayed unless fluorotic symptoms had appeared.

Symptoms of fluorosis usually began to appear between the 22nd and 26th days, and generally developed into a well defined syndrome of fluorine toxicosis within 10 to 15 days. Lot V which also received fluorine appears to disrupt the trend of the data in the first three lots. However, two animals from this lot were removed in the early stages of the development of fluorosis and it was found that the ascorbic acid values of the suprarenal gland were well above the average of our control lot and contained respectively, 0.815 and 0.70 mg. of ascorbic acid per gm. of fresh tissue. If these two animals are disregarded the ascorbic acid content decreases from 0.528 mg. per gm. to an average of 0.353 mg. per gm. of tissue for the lot which may be considered at least near the border line of scurvy. 12 cc. of orange juice maintained the ascorbic acid content of the suprarenal gland but did not prevent the appearance of the clinical symptoms of fluorosis. These symptoms of fluorosis simulating those of scurvy were in most cases present at the time the animals were sacrificed in this lot.

We regret that insufficient material for analysis makes a clear cut conclusion concerning the influence of fluorosis and scurvy upon the glutathione content of the suprarenal gland exceedingly hazardous. The glutathione content seemed to be increased in the fluorine-poisoned animals. Since the glutathione was calculated from the excess iodine above that required to titrate the vitamin C, or ascorbic acid, the latter when considerably reduced in amount, as in the case of fluorosis, materially influences the amount of the glutathione present in the gland.

Table III gives the range of values and the averages for the rate of respiration and the cyanide inhibition for the liver tissue as measured in glucose, lactate, and succinate substrates and in plain buffer. When the average results are determined in glucose substrate little difference is observed among the various lots. The oxygen uptake averages close to 870 c.mm. per gm. per 2 hours and the cyanide inhibition is about 60 per cent. The oxygen uptake for Lot VII is somewhat lower than for the other groups. Similarly the average results for oxygen uptake and cyanide inhibition measured in lactate substrate, succinate substrate, and in plain buffer show little variation among the different lots, though a difference is observed in the values obtained with the various substrates.

TABLE III

Oxygen Uptake of Liver Tissue from Guinea Pigs. Measured at 37° in Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported in c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	O_2 uptake, no substrate	Per cent CN inhibition	O_2 uptake, glucose	Per cent CN inhibition	O_2 uptake, lactate	Per cent CN inhibition	O_2 uptake, succinate	Per cent CN inhibition
Lot I. Basal ration (scorbutic)............	915 (2)*	55	950 (8)	60	1120 (7)	45	1980 (3)	31
Lot II. Basal + 3 cc. orange juice...........	990 (2)	50	800 (8)	49	1200 (7)	51	1940 (1)	
Lot III. Lot II diet + NaF..................	890 (2)	52	850 (7)	52	1088 (5)	45	1680 (2)	21
Lot IV. Basal + 6 cc. orange juice...........	840 (1)	52	830 (3)	62	990 (4)	43	1710 (1)	15
Lot V. Basal + 6 cc. orange juice + NaF....	820 (1)	56	930 (4)	70	1120 (3)	52	1850 (2)	22
Lot VI. Basal + 12 cc. orange juice + NaF....			850 (4)	56	1150 (4)	50		
Lot VII. Basal + crystalline ascorbic acid....			760 (3)	52				

* The numbers in parentheses indicate the number of animals used for calculating the values obtained.

The results of the rate of oxygen consumption and cyanide inhibition determined in plain buffer are very similar to the values obtained in glucose buffer. It appears as though the addition of glucose *in vitro* does not increase the rate of oxygen consumption of liver tissue when measured over a period of 2 hours.

The data presented in Table III indicate that scurvy and fluorine poisoning have relatively little effect on the rate of oxygen uptake and the cyanide inhibition of liver tissues of guinea pigs as measured under the conditions described in this paper. These results do not confirm the conclusions made by Harrison (7), that the rate of oxygen uptake of liver tissue from scorbutic guinea pigs is lower than that of liver from normal animals. However, the results obtained are not strictly comparable with Harrison's (7) data, since he made his measurements in plain phosphate buffer on slices of liver tissue rather than on minced tissue and in an atmosphere of oxygen rather than of air.

TABLE IV

Effect of Ascorbic Acid (Karrer), in Vitro, on Oxygen Uptake of Liver Tissue from Guinea Pigs. Measured at 37° in Glucose-Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported as c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	Guinea pig No.	No ascorbic acid added		0.12 mg. ascorbic acid added		Change on addition of ascorbic acid	
		O_2 uptake	Cyanide inhibition in per cent of total	O_2 uptake	CN inhibition in per cent of total	Absolute	Per cent
Lot I. Basal ration (scorbutic)	9*	980	54	1040	46	+60	+6
	10*	850	55	1050	74	+200	+24
Lot II. Basal + 3 cc. orange juice	8*	1300	55	820	34	−480	−37
	9	900	54	720	11	−180	−20
	10	740	51	770	52	000	00
Lot III. Lot II diet + NaF	8	1020	73	1050	66	+30	+3
	9	860	40	940	40	+80	+9
Lot VII. Basal + crystalline ascorbic acid							
(Link)	1	710	41	780	53	+70	+10
(Karrer)	1	640	70	640	47	00	00
(Bauman)	1	930	46	990	58	+60	+6

* These determinations were made in plain phosphate-Ringer's solution buffer; no added substrate.

Harrison also reported that the *in vitro* addition of ascorbic acid brought about an increase in the oxygen uptake of scorbutic liver slices. Even though the results reported in Table III show little difference in the rate of oxygen consumption of the liver tissue from the scorbutic animals as compared with the normal animals, it would be of interest to observe the effect of *in vitro* addition of ascorbic acid on the oxygen uptake measurements, especially since a diminution of vitamin C in the liver tissue was detected in the scorbutic group (Lot I). A few experiments on the effect of *in vitro* addition of 0.12 mg. of ascorbic acid on the rate of oxygen consumption of liver tissue have been carried out and the results are given in Table IV. These determinations as well as all of the other determinations of oxygen uptake on liver tissue were made on samples of liver weighing in the region of 200 mg. The addition of 0.12 mg. of ascorbic acid represents a concentration of 5 times the average amount found in 200 mg. of normal liver tissue. Harrison in his experiments added 0.25 mg. of ascorbic acid and used tissue slices averaging 200 mg. From Table IV it is readily seen that the effect of addition of ascorbic acid is not very pronounced and is quite variable. There appears to be a slight tendency to increase the respiration of the liver from the scorbutic guinea pigs and not to affect or to decrease slightly the respiration of the liver from the normal guinea pigs. The data presented are insufficient from which to draw definite conclusions.

Table V gives the average time required for extracts of the liver tissue to decolorize methylene blue in the presence of various substrates and the effect of cyanide on the decolorization time.

Examination of Table V readily shows that the oxidation of succinate occurs much more rapidly than does the oxidation of lactate, and the oxidation of lactate occurs more rapidly than the oxidation of glucose or formate. The decolorization time of methylene blue is not decreased significantly by the addition of glucose or formate as compared to the decolorization time in plain buffer. A few experiments not listed in Table V were performed with tartaric acid, maleic acid, arabinose, and xylose as substrates. With none of these substrates was the decolorization time decreased as compared to the time required when no substrate (plain buffer) was used, and with tartaric acid and maleic acid a definite inhibiting effect on the decolorization of methylene blue was observed. In all cases cyanide increased the time required for decolorization.

Comparing the decolorization time of the liver extracts from the various groups of guinea pigs, one observes no great difference.

The scorbutic group (Lot I) seems to be slightly more active in decolorizing the dye. This effect is observed with all of the substrates and also with the plain buffer. Fluorine poisoning tends to slow down the decolorization of methylene blue in succinate substrate as may be seen when the fluorine-fed lots (Lots III and V) are compared with their normal control lots (Nos. II and IV). With Lot VI, the guinea pigs fed on the high orange juice level, there is a definite trend toward more rapid decolorization of methylene blue in all of the substrates used. Even though the guinea pigs in Lot VI received fluorine, the high level of orange juice fed (12 cc. per guinea pig per day) seemed to counteract the inhibiting effect observed with succinate buffer in the fluorine-fed lots on the lower levels of orange juice (Lots III and V).

TABLE V

Decolorization of Methylene Blue by Extracts of Livers from Guinea Pigs. Experiments Run at 37°, pH 7.3, in Thunberg Tubes

Values are reported in minutes for 80 per cent decolorization.

	No. of guinea pigs	Average decolorization time of methylene blue in min. Various substrates				
		Succinate	Lactate	Glucose	Formate	No substrate
Lot I. Basal ration (scorbutic).	8	6	20	43	55	64
" II. " + 3 cc. orange juice.	9	7	29	64	61	72
Lot III. Lot II diet + NaF.	9	8	27	51	38	58
" IV. Basal + 6 cc. orange juice.	5	6	40	56	86	90
" V. " + 6 " " " + NaF.	5	7	30	67	83	88
Lot VI. Basal + 12 cc. orange juice + NaF.	4	5	16	38		

The indophenol oxidase content was estimated by the ability of the liver extracts to cause the oxidation of the Nadi reagent to indophenol. The researches of Keilin (12–14) have shown that indophenol oxidase is the active agent in the oxidation of cytochrome and thus it assumes an important part in the oxidative mechanisms of the tissues. It was observed that the relative concentration of indophenol oxidase is distinctly decreased in the liver extracts prepared from the scorbutic guinea pigs and the guinea pigs in the fluorine-fed groups as compared with the two control lots (Lots II and IV). When the oxidase tests were conducted on extracts heated at 45° for ½ hour, it is observed that the general effect was to decrease the sensitivity of the test, in other words the indophenol oxidase of the liver tissue is slightly heat-labile when held at 45° for ½ hour.

Since a decrease in the indophenol oxidase content as measured by the oxidation of the Nadi reagent was observed in the liver extracts from the scorbutic and the fluorine-fed animals, it was of interest to observe the cytochrome spectrum. Slices of liver, kidney, and heart tissue were reduced with sodium hydrosulfite, placed between two glass slides, and examined with a microspectroscope for the cytochrome absorption spectrum. In tissues from animals in all lots a distinct absorption spectrum of reduced cytochrome was observed. By such simple qualitative examination of the absorption spectrum little indication is given as to the relative amount of cytochrome; however, it could readily be observed that the concentration of cytochrome was greatest in heart tissue, though there was no decided decrease in any of the components of the absorption spectrum in the tissues from guinea pigs in the various lots.

Because ascorbic acid is concentrated in the suprarenal gland and because in scurvy the amount of ascorbic acid in this gland is markedly decreased, it would be of interest to compare the rates of oxygen consumption of suprarenal tissue from scorbutic and normal animals and with fluorine-poisoned animals.

The literature contains few reports of the rate of oxygen consumption of the suprarenal tissue. Von Euler, Myrbäck, and Larsson (5) reported that the oxygen uptake of the medulla of the suprarenal gland of beef cattle was greater than that of the cortex. Similar observations on dairy cows were recorded by Phillips and Stare (19). No observations on the rate of oxygen consumption of the suprarenal tissue from guinea pigs were found in the literature.

In Table VI are given the range of values and the averages for the rate of oxygen uptake and the cyanide inhibition of the suprarenal tissue as measured in glucose-phosphate-Ringer's solution buffer. When the average rate of oxygen consumption of the tissue from the scorbutic group (Lot I) is compared with that of the two normal groups (Lots II and IV), it is quite obvious that scurvy produces a marked decrease in the oxygen consumption of the tissue of the suprarenal gland. The average rate of oxygen uptake of the tissue from the scorbutic group was 455 c.mm. per gm. per 2 hours, a decrease of 55 per cent as compared with the controls receiving 3 cc. of orange juice per day (Lot II), and compared with the controls receiving 6 cc. of orange juice per day (Lot IV) a decrease of 66 per cent. A decrease in the rate of respiration of the same order is observed in the average values for

TABLE VI

Oxygen Uptake of Suprarenal Tissue from Guinea Pigs. Measured at 37° in Glucose-Phosphate-Ringer's Solution Buffer, pH 7.3

Values are reported as c.mm. of O_2 per gm. of tissue, wet weight, per 2 hours.

	No. of guinea pigs	Average values	Average cyanide inhibition in per cent of total
Lot I. Basal ration (scorbutic)	8	455	10
“ II. “ + 3 cc. orange juice	8	1000	33
“ III. Lot II diet + NaF	8	540	45
“ IV. Basal + 6 cc. orange juice	4	1335	69
“ V. “ + 6 “ “ “ + NaF	4	525	100
“ VI. “ + 12 “ “ “ + NaF	4	605	91
“ VII. “ + crystalline ascorbic acid			
(Link)	2	930	
(Karrer)	2	1135	
(Bauman)	2	680	

the suprarenal tissue from the three fluorine-fed groups (Lots III, V, and VI). The suprarenal tissue from the guinea pigs in Lot VII gives a rate of oxygen consumption equally comparable with the values of the two normal groups. The low average value for the two guinea pigs in Lot VII is considerably lower than for the two other ascorbic acid-fed groups. This low figure is due largely to the low result of one of the guinea pigs which for some reason objected strenuously to swallowing the ascorbic acid, and it is probably not due to a less active ascorbic acid preparation.

When the effect of cyanide on the rates of oxygen uptake of the suprarenal tissue is compared, considerable difference is observed among the various lots. Cyanide produced only a 10 per cent inhibition in the scorbutic group. If ascorbic acid functions prominently in the oxidative metabolism of the suprarenal gland, one would expect little cyanide inhibition on the rate of oxygen uptake of tissue from scorbutic animals, since the ascorbic acid, even though its reversible oxidation-reduction characteristic is inhibited by cyanide, is practically nil in the suprarenal tissue from scorbutic guinea pigs. When Lots II and III are compared, both of which were on the lower level of orange juice and one of which received fluorine, the cyanide inhibition is found to increase in the fluorine-fed group. This same phenomenon is observed in comparing Lots IV and V which were fed with the higher level of orange juice. Likewise, in the other fluorine-fed group (Lot VI) a high cyanide inhibition was observed. It should be mentioned that a considerably greater variation was obtained between the cyanide inhibition values of the suprarenal tissue of the individual guinea pigs of the various lots as compared with the variation in the rates of oxygen uptake. Too much emphasis should not be placed on these findings until they are repeated, though it appears that in fluorine poisoning there is a tendency to increase the cyanide-inhibited respiration factor, and curiously, with the exception of Lot III, the extent of the cyanide inhibition is roughly proportional to the amount of ascorbic acid found in the suprarenal tissue.

Since such a marked decrease was observed in the rates of oxygen uptake of the suprarenal tissue of scorbutic and fluorine-poisoned guinea pigs as compared with normal controls and because the ascorbic acid content of this tissue is very low in scorbutic animals, it would be of interest to study the effect of *in vitro* addition of ascorbic acid on the rate of oxygen consumption. A few experiments of this order were performed. It was observed that the rate of respiration of the suprarenal tissue from two scorbutic guinea pigs (only two animals available) was increased very materially by the *in vitro* addition of ascorbic acid. With animals in Lots II and III the effect observed was variable. With Lot VII the same phenomenon was observed as was with the *in vitro* addition of ascorbic acid to the liver tissue from this lot; namely, the *in vitro* addition of the same preparation of ascorbic acid as fed to the animals (Karrer's preparation) did not cause an increase in the rate of oxygen consumption; however, when this ascorbic acid was added to the suprarenal tissue obtained from the guinea pigs in Lot VII, which were fed other ascorbic acid preparations, a considerable increase in the rate of oxygen uptake was observed. The results on the effect of *in vitro* addition of ascorbic acid on the rate of oxygen uptake of suprarenal tissue were too few in number to warrant drawing any generalizations, but they point to an interesting study.

DISCUSSION

The nature of the oxidative mechanisms involved in the chemical reactions constituting tissue respiration is extremely complex, and our present knowledge of the subject is quite meager. The facts emerging from the experimental data reported in this paper indicate that the activity of certain cellular oxidative mechanisms is intimately associated with the syndromes of scurvy and fluorine poisoning. A correct interpretation of the data relative to scurvy and fluorine toxicosis is limited by the extent of the experimentation and by the limited knowledge of the intracacies of the respiration of animal tissues.

The results obtained with the liver tissue were consistent and a logical interpretation of them may be presented. The total respiration as represented by the rate of oxygen consumption remained fairly constant in the animals from the various groups. The inhibiting effect of cyanide on the rate of oxygen uptake was similarly quite constant. Qualitative tests for indophenol oxidase with the Nadi reagent were markedly less in the livers of the scorbutic and the fluorine-poisoned animals as compared with the concentration found in the livers from the normal control animals. According to Keilin (14) indophenol oxidase functions in oxidizing the cytochrome reduced in the dehydrogenation of the tissue metabolites. A decrease in the indophenol oxidase content of a tissue would lower the effectiveness of the oxidase-cytochrome system and unless the activity of other oxidative systems was not increased one would expect the rate of oxygen consumption to be diminished. Since indophenol oxidase is inactivated by cyanide any decrease in respiration from a lowering of the oxidase could be detected by a lowering of the cyanide-inhibited respiration fraction. It was found that the rate of oxygen consumption and the cyanide inhibition of the rate of oxygen consumption of the liver tissue from scorbutic and fluorine-poisoned animals were not significantly changed from the values obtained with tissue from normal animals, therefore the experimentally observed decrease in indophenol oxidase must have been compensated for by an increase in the activity of some other component of the cyanide-inhibited respiration fraction.

The glutathione content of liver tissue increased in scurvy and in fluorine toxicosis. In normal tissue respiration glutathione may function in a manner similar to cytochrome. The oxidation of reduced glutathione is dependent on metals which are poisoned by cyanide, thus glutathione is a part of the cyanide-inhibited respiration fraction. The observed increase in glutathione content suggests that it has assumed the function formerly performed by the oxidase-cytochrome system. If this assumption were true it would be predicted that the total respiration would remain the same and that the fraction inhibited by cyanide would remain the same. The data show this to be the case. In so far as these results are concerned no other component seemed to be involved in the respiration of the liver tissue. Lowering the vitamin C content of the liver by scurvy produced no marked changes in oxygen uptake. The addition of pure ascorbic acid to minced liver did not significantly change the oxygen uptake in tissue obtained from either the scorbutic, the fluorine-poisoned, or the normal animals.

The results with the decolorization of methylene blue by extracts prepared from the livers from the animals on the different diets do not differ enough to draw any correlations with the syndromes of scurvy or fluorine poisoning. These results indicate that apparently scurvy or fluorine toxicosis has no effect on the hydrogen-activating systems.

In the case of the suprarenal gland the quantity of material available for study from each animal was so small as to handicap seriously a complete study such as that made upon the liver. No material was available for testing this tissue with Nadi's reagent. The quantity available for iodine titration was too meager for reliable results. Thus the lack of complete and accurate data relative to these two substances is somewhat disappointing. Had complete data been possible perhaps it would permit of a more rational interpretation.

The results obtained with the suprarenal gland in other respects are extremely interesting. The most likely explanation for the hypertrophy of the gland, it seems to us, is a compensatory effort on the part of the organism to maintain an indispensable function. We are not unmindful of the somewhat prevalent opinion that the suprarenal function is concerned with water metabolism and that the increase in weight might be due to additional fluids in the gland. Regardless of what the ultimate cause may be shown to be, we are chiefly concerned with the fact that its weight does increase.

Other pertinent facts concerning the suprarenal gland have been obtained. The oxygen uptake fraction inhibited by cyanide is very low in the case of scurvy and high in fluorine poisoning. Apparently the efficiency of the gland is decreased in either syndrome. As to the mechanism involved in the respiration of the suprarenal gland, we cannot state. It seems not unlikely that the following offered merely as a suggestion may in part rightfully interpret our results. The ascorbic acid content of the suprarenals decreases relatively and absolutely in scurvy and fluorine toxicosis. Since the addition of ascorbic acid to suprarenal tissue *in vitro* caused the return of normal oxygen uptake, it suggests immediately that ascorbic acid seems to function as an oxidation-reduction intermediary agent. The cyanide action upon oxygen uptake would support this view. The cyanide-inhibited portion of respiration is decreased in scurvy, perhaps due to the decrease in ascorbic acid. This would be in conformity with Szent-Györgyi's (24) finding that the oxidation-reduction activity of ascorbic acid was inhibited by cyanide. In the fluorine-fed animals the CN-inhibited portion of respiration was definitely increased. The vitamin C content of the suprarenal gland in these cases, while appreciably lowered, was not depleted to the extent that it was in the case of scurvy. If this quantity of vitamin C were functional it might account for the CN inhibition of respiration. However, the slight response of glutathione in the fluorine-fed animals would also tend to increase this portion of the respiration of the gland. On the other hand large quantities of orange juice fed in Lot VI (12 cc. daily) indicate that the ascorbic acid content of the gland can be maintained in fluorosis and yet the total O_2 uptake is significantly decreased from the normal. Thus the evidence in the case of fluorine points to one of two alternative conclusions. Either the fluorine inactivates the ascorbic acid as far as the animal economy is concerned, or the fluorine specifically inhibits an enzyme system in which ascorbic acid is involved. The latter point of view seems more in keeping with the known facts concerning fluorine action in the body. Thus it would seem plausible to suggest that ascorbic acid has a function in the respiration of the suprarenal gland. In scorbutus the ascorbic acid eliminates the major portion of this system while in fluorosis some enzyme link of the oxidative system is inhibited.

Other observations made in these studies require some comment. It seems not unimportant that the ascorbic acid content of the anterior lobe of the hypophysis should follow the general relationship to scurvy and fluorosis that were found to exist in the suprarenal gland. The known important hormonal function of this gland requires no comment. Should the relationship to cellular metabolism be found to follow those shown to exist for the suprarenal gland, the action of fluorine and ascorbic acid would be further elucidated. The similarity of the action of fluorine and scurvy are again demonstrated. The symptomatic likeness was mentioned previously. In these studies the effect of fluorine and scurvy upon the liver was alike in that both syndromes eliminated the indophenol oxidase in so far as this could be detected by Nadi's reagent. Both diseases cause the suprarenal gland to hypertrophy. The oxygen uptake of the suprarenal tissue was uniformly decreased in both diseases approximately 50 per cent. The CN-inhibited portion of the total respiration was dissimilarly affected but the likelihood of this result being caused by a disturbance in the same system may explain this difference. The ascorbic acid content was decreased in scurvy and fluorosis. In the latter the decrease was much less marked but nevertheless uniformly lowered. There was a tendency for glutathione to increase in the fluorine-fed groups as compared to scorbutic and normal control animals. In these respects chronic fluorosis and scurvy have been found to exert much the same physiological response in the liver and suprarenal gland. The symptoms of scurvy cannot be directly associated with the decrease of ascorbic acid in the liver but may be directly associated with the decrease of ascorbic acid in the suprarenal gland.

SUMMARY

1. Ascorbic acid is widely distributed in the tissues of the normal guinea pig and is concentrated in the anterior lobe of the pituitary and in the suprarenal gland. It may be decreased to subfunctional levels and increased to relatively high values by varying the ascorbic acid content of the diet.
2. The rate of oxygen uptake of liver tissue and the inhibiting effect of cyanide is not significantly different for liver tissue obtained from scorbutic, fluorine-poisoned, or normal guinea pigs.
3. Decolorization time of methylene blue by extracts prepared from liver tissue from scorbutic, fluorine-poisoned, or normal guinea pigs is not significantly different.
4. The rate of oxygen uptake, the inhibiting effect of cyanide, and the decolorization time of methylene blue are all dependent on the substrate in which they are measured. Succinate is most rapidly oxidized, lactate next, and formate and glucose differ little from plain buffer solution without added substrate.
5. The indophenol oxidase content as qualitatively estimated by the coloration of the Nadi reagent is markedly diminished in the liver tissue of scorbutic and fluorine-fed guinea pigs as compared with normal controls, and the glutathione content increases, suggesting a compensating action on the part of glutathione.
6. Cytochrome is present in the heart, kidney, and liver tissue from scorbutic, fluorine-poisoned, and normal guinea pigs.
7. The rate of oxygen uptake of the suprarenal tissue from scorbutic and fluorine-poisoned guinea pigs is about half that found in the tissue from normal control animals. In the case of two scorbutic animals (only two animals available) the oxygen uptake was increased to the normal value by the *in vitro* addition of ascorbic acid. The cyanide-inhibited fraction of the oxygen uptake of the suprarenal tissue is very low in scorbutic animals, high in fluorine-poisoned animals, and in general is proportional to the amount of vitamin C in the tissue.
8. Ascorbic acid is definitely associated with a considerable portion of the cyanide-inhibited intracellular respiration of the suprarenal gland of the guinea pig.
9. The similarity between scurvy and fluorosis is thus confirmed and extended by these researches. The evidence obtained indicates strongly that ascorbic acid deficiency and the deleterious

effect of chronic fluorine toxicosis result primarily from disturbances in specific phases of cellular respiration.

We wish to express our thanks and appreciation to Dr. P. Karrer, Dr. E. J. Bauman, and Dr. K. P. Link for supplying us with the crystalline preparations of ascorbic acid used in these studies.

BIBLIOGRAPHY

1. Bessey, O. A., and King, C. G., *J. Biol. Chem.*, **103**, 687 (1933).
2. Birch, T. W., and Dann, W. J., *Nature*, **131**, 469 (1933).
3. Birch, T. W., Harris, L. J., and Ray, S. N., *Nature*, **131**, 273 (1933).
4. von Euler, H., and Malm, M., *Ark. Kemi, Mineral. o. Geol.*, **11 B**, Nos. 6–9 (1932–33).
5. von Euler, H., Myrbäck, K., and Larsson, H., *Z. physiol. Chem.*, **217**, 1 (1933).
6. Harris, L. J., and Ray, S. N., *Biochem. J.*, **27**, 2006 (1933).
7. Harrison, D. C., *Biochem. J.*, **27**, 1501 (1933).
8. Hartman, F. A., *Ann. Int. Med.*, **7**, 6 (1933).
9. Hopkins, F. G., and Elliott, K. A. C., *Proc. Roy. Soc. London, Series B*, **109**, 58 (1931).
10. Huszak, I., *Z. physiol. Chem.*, **222**, 229 (1933).
11. Karrer, P., and Zehender, F., *Helv. chim. acta*, **16**, 701 (1933).
12. Keilin, D., *Proc. Roy. Soc. London, Series B*, **98**, 312 (1925).
13. Keilin, D., *Proc. Roy. Soc. London, Series B*, **104**, 206 (1929).
14. Keilin, D., *Proc. Roy. Soc. London, Series B*, **106**, 418 (1930).
15. Lockwood, J. E., and Hartman, F. A., *Endocrinology*, **17**, 501 (1933).
16. Mosonyi, J., and Rigó, L., *Z. physiol. Chem.*, **222**, 100 (1933).
17. Phillips, P. H., *Proc. Am. Soc. Biol. Chem.*, **8**, lxxix (1933); *J. Biol. Chem.*, **100**, lxxix (1933).
18. Phillips, P. H., and Chang, C. Y., *J. Biol. Chem.*, **105**, 405 (1933).
19. Phillips, P. H., and Stare, F. J., *J. Biol. Chem.*, **104**, 351 (1934).
20. Purr, A., *Biochem. J.*, **27**, 1703 (1933).
21. Sato, R., and Ohata, S., *J. Biochem.*, Japan, **14**, 325 (1931).
22. Söderström, N., and Törnblom, N., *Skand. Arch. Physiol.*, **66**, 67 (1933).
23. Svirbely, J. L., *Biochem. J.*, **27**, 960 (1933).
24. Szent-Györgyi, A. V., *Biochem. J.*, **22**, 1387 (1928).
25. Szent-Györgyi, A. V., *Deutsch. med. Woch.*, **58**, 582 (1932).
26. Thunberg, T., *Skand. Arch. Physiol.*, **40**, 1 (1920).
27. Wolff, R., and Manjean, S., *Compt. rend. Soc. biol.*, **111**, 624 (1932).

DERIVATIVES OF GLUCURONIC ACID

IV. THE SYNTHESIS OF α- AND β-TETRAACETYLGLUCURONIC ACID METHYL ESTER AND OF 1-CHLOROTRIACETYLGLUCURONIC ACID METHYL ESTER

BY WALTHER F. GOEBEL AND FRANK H. BABERS

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, May 7, 1934)

In Paper III of this series (1) the preparation of diacetylchloroglucuron was described. At that time it was hoped that this derivative of *d*-glucuronic acid could be used for the preparation of synthetic glucuronides. It has since been found that when diacetylchloroglucuron is condensed with methyl alcohol or with nitrobenzyl alcohol in the presence of silver carbonate or silver oxide crystalline products are obtained which have the correct analysis for the corresponding diacetyl glycosides of glucuron. These compounds, however, have certain properties which differentiate them from true glycosides. Not only do these derivatives of glucuron reduce Fehling's solution, but in certain other respects they behave as though an aldehydic or ketonic group were present within the molecule. Although the structures of these substances have not yet been established, it seems, from certain evidence at hand, that their reducing properties cannot be attributed to a terminal aldehydic group.

In order to secure an acetohalogen derivative of *d*-glucuronic acid which might be used for the synthesis of glucuronides, the acid itself was chosen as the source material. The silver salt of glucuronic acid can be formed by stirring a cold aqueous solution of the acid with a suspension of silver carbonate. The methyl ester of *d*-glucuronic acid is prepared by allowing the silver salt to interact with methyl iodide. Although the methyl ester of *d*-glucuronic acid thus formed is not crystalline, the amorphous derivative yields a mixture of crystalline α- and β-tetraacetylglucuronic acid methyl ester on acetylation either with acetic anhydride and zinc chloride or with pyridine and acetic anhydride. These isomeric acetyl derivatives may be separated by fractional crystallization. After six recrystallizations the α form of tetraacetylglucuronic acid methyl ester melts at 111–112° and has a specific rotation of +98.0° in chloroform. The β isomer, on the other hand, melts at 178° and shows a specific rotation of +8.7° when taken in the same solvent. In their present state of purity the difference in the molecular rotations of α- and β-tetraacetylglucuronic acid methyl ester is 33,500.

When either α- or β-tetraacetylglucuronic acid methyl ester is treated with acetyl chloride and hydrogen chloride, the derivatives are, in each instance, converted into the same crystalline 1-chlorotriacetylglucuronic acid methyl ester. The halogen derivative is levorotatory, and shows a specific rotation of −16.7° when taken in chloroform.

Triacetylchloroglucuronic acid methyl ester is a stable derivative, and may be kept in a desiccator for a long period of time without decomposition. The substance is slightly less soluble in chloroform and considerably more soluble in ether than is diacetylchloroglucuron. When shaken with anhydrous methyl alcohol in the presence of silver carbonate, triacetylchloroglucuronic acid methyl ester yields a crystalline glucuronide which does not reduce Fehling's solution. The synthesis and properties of this glycoside will be described in a later communication. In the present paper the method is given for the preparation of α- and β-tetraacetylglucuronic acid methyl ester and of 1-chlorotriacetylglucuronic acid methyl ester.

EXPERIMENTAL

Silver Glucuronate—A mixture of *d*-glucuronic acid and its lactone, glucuron, was obtained by the hydrolysis of the borneol glycoside of *d*-glucuronic acid according to the method of Quick (2). In order to prepare the silver salt of glucuronic acid from this mixture it is necessary first to convert the lactone into glucuronic acid. For this purpose, therefore, a preliminary analysis is necessary in order to determine the percentage of lactone and acid in the mixture. The analysis is performed in the usual way by titrating a weighed sample with 0.1 N NaOH at 0°, with phenolphthalein as indicator. The percentage of free glucuronic acid is then calculated, and the percentage of glucuron is determined by difference.

The silver salt of glucuronic acid was prepared in the following manner. 25 gm. of the glucuronic acid-lactone mixture were dissolved in 50 cc. of warm water and 0.2 cc. of 1 per cent phenolphthalein solution was added. The free glucuronic acid in the solution was neutralized with the theoretical quantity of 0.4 N $Ba(OH)_2$ solution. Sufficient base to neutralize the remaining lactone was now carefully added, and at such a rate that at no time did the solution become definitely alkaline to the indicator. The period of time required for the addition of the second portion of the base is approximately 2 hours. After the total volume of base had been added, the solution of the barium salt of glucuronic acid was cooled to 0°. The barium was quantitatively removed with 3 N H_2SO_4 and the precipitated barium sulfate was separated by centrifugation at 0°. The solution of glucuronic acid was decolorized with charcoal and carefully concentrated to 100 cc. *in vacuo*.

The cold solution of glucuronic acid was now added to an aqueous suspension of 2 moles of freshly precipitated silver carbonate. The mixture was stirred at 0° for 2 hours, and then centrifuged at 0° to remove excess silver carbonate. The pale yellow solution of the silver salt of glucuronic acid was poured into 7 volumes of chilled redistilled methyl alcohol. The precipitated silver salt was filtered on a large sintered glass funnel, washed with methyl alcohol and ether, and dried for 3 or 4 days over P_2O_5 in a brown glass vacuum desiccator. 32.0 gm. of the amorphous silver salt of glucuronic acid were recovered. The salt may be crystallized by cooling a concentrated aqueous solution to 0°.

The alcoholic filtrate from the silver salt, when concentrated *in vacuo*, yielded a small amount of a white crystalline product which proved to be the silver salt of glucuronic acid in crystalline form. This material was dried in the air and analyzed.

Analysis—5.225 mg. substance: 2.455 mg. AgCl
$C_6H_9O_7Ag$. Calculated, Ag. 35.66; found, Ag 35.38
$[\alpha]_D^{28} = (+0.21° \times 100)/1.093 = +19.2°$ (in water)

Mutarotation was not observed.

Glucuronic Acid Methyl Ester—30 gm. of silver glucuronate were suspended in 200 cc. of methyl iodide and shaken until the precipitate no longer showed the presence of water-soluble silver ion. The mixture was filtered and the precipitate washed with ether. The gummy residue of silver iodide, containing the methyl ester of glucuronic acid, was extracted with water and filtered. The aqueous filtrate, which contained a certain amount of free glucuronic acid, was now neutralized with barium carbonate, filtered, and concentrated *in vacuo.* 300 cc. of absolute ethyl alcohol were added and the precipitated barium glucuronate was separated by filtration. The alcoholic filtrate, containing the methyl ester of glucuronic acid, was concentrated to a syrup *in vacuo.* The residue was dissolved in 100 cc. of alcohol and again concentrated. This procedure was repeated four times in all in order to remove water. The colorless amorphous residue, the methyl ester of glucuronic acid, was dried in a vacuum until its weight remained constant. 15.3 gm. of ester were recovered.

Analysis—3.611 mg. substance: 4.194 mg. AgI

$C_7H_{12}O_7$. Calculated, OCH_3 14.90; found, OCH_3 15.34

$[\alpha]_D^{23} = (+1.33° \times 100)/(2 \times 1.492) = +44.6°$ (in water)

Mutarotation was not observed.

The preparation of the methyl ester of glucuronic acid by the method described is always accompanied by the formation of considerable quantities of free glucuronic acid. This may be due to the fact that the amorphous silver salt of glucuronic acid, which on analysis is found to be somewhat low in silver content, contains approximately 0.5 mole of water. The silver salt of glucuronic acid tends to darken when kept in a vacuum over P_2O_5 in a brown glass desiccator. It is therefore not advisable to dry the salt longer than 4 to 5 days before converting it into the methyl ester. Since within this time the last traces of water cannot be removed, its presence probably causes the formation of free glucuronic acid when the silver salt is converted to the ester.

The methyl ester of glucuronic acid may also be prepared by shaking the silver salt in methyl alcohol together with 1.1 moles of methyl iodide. Under these conditions approximately twice as much free glucuronic acid is formed as when the reaction is carried out in the presence of pure methyl iodide. If the silver salt is shaken with an excess of methyl iodide (2 moles) in a neutral solvent such as toluene, the reaction takes several weeks to come to completion. In order to improve the yields of glucuronic acid methyl ester, attempts are being made at present to esterify the carboxyl group by means of diazomethane.

Acetylation of Methyl Ester of Glucuronic Acid with Acetic Anhydride and Zinc Chloride—11.3 gm. of the methyl ester of glucuronic acid were treated at 0° with 70 cc. of acetic anhydride in which were dissolved 7 gm. of anhydrous zinc chloride. The ester slowly dissolved. The mixture was gradually warmed to room temperature, and finally to 50° for 25 minutes. The acetic anhydride was removed by distillation *in vacuo*. The residue in the flask was dissolved as completely as possible in 100 cc. of chloroform, 100 cc. of water were added, and the mixture was transferred to a separatory funnel. The solution of the acetyl derivative was now washed at 0° with 0.2 N $NaHCO_3$ and finally with ice water. After dehydrating the chloroform solution with sodium sulfate, the solvent was removed by distillation *in vacuo*. 75 cc. of ethyl alcohol were added, and soon after the oily residue had dissolved crystallization of the tetraacetate of the methyl ester of glucuronic acid commenced. The flask was cooled to 0° and the crystals were removed by filtration. 6.5 gm. of substance were recovered. The mother liquors were concentrated to 40 cc. *in vacuo*. After standing overnight the crystals which separated from the solution were filtered. 5.3 gm. were recovered.

The first crystals to separate from the alcoholic solution proved to be the β form of the tetraacetylglucuronic acid methyl ester. The substance was recrystallized four times from alcohol. The material melted sharply at 178° (corrected). The substance is difficultly soluble in ethyl alcohol, and separates from hot solutions as glistening crystals 1 to 2 cm. in length. The substance was analyzed for its acetyl content by the method of Pregl (3).

Analysis—7.291 mg. substance: 5.34 cc. N/70 NaOH
$C_{15}H_{20}O_{11}$. Calculated, $COCH_3$ 45.75; found, $COCH_3$ 45.00
4.269 mg. substance: 2.658 mg. AgI
$C_{15}H_{20}O_{11}$. Calculated, OCH_3 8.25; found, OCH_3 8.23

$$[\alpha]_D^{24} = (+0.18° \times 100)/(2 \times 1.034) = +8.7° \text{ (in chloroform)}$$

The second crystalline derivative which separated from the original reaction mixture was found to be the α form of the tetraacetylglucuronic acid methyl ester. The substance was recrystallized six times from ethyl alcohol, a solvent in which it is far more soluble than is the β isomer. After the sixth crystallization the compound melted at 111–112° (corrected) and showed the following analysis and specific optical rotation.

Analysis—7.161 mg. substance: 5.36 cc. N/70 NaOH

$C_{15}H_{20}O_{11}$. Calculated, $COCH_3$ 45.75; found, $COCH_3$ 45.95

3.230 mg. substance: 2.055 mg. AgI

$C_{15}H_{20}O_{11}$. Calculated, OCH_3 8.25; found, OCH_3 8.41

$$[\alpha]_D^{24} = (+2.62° \times 100)/(2 \times 1.337) = +98.0° \text{ (in chloroform)}$$

Acetylation of Methyl Ester of Glucuronic Acid with Acetic Anhydride and Pyridine—17.9 gm. of the methyl ester of glucuronic acid were dissolved in 75 cc. of pyridine and 50 cc. of acetic anhydride at 0°. The mixture was allowed to stand for 3 hours in the ice box. The solvent was evaporated by distillation *in vacuo* until crystals of β-tetraacetylglucuronic acid methyl ester separated from the solution. The mixture was cooled to 0° and filtered. The crystals were washed with small portions of chilled alcohol and ether. 10.9 gm. of pure β-tetraacetylglucuronic acid methyl ester were recovered. The filtrate from these crystals was now concentrated to a syrup *in vacuo*, and the residue taken up in ethyl alcohol. On cooling crystals of the α isomer separated from the solution. The product was filtered and washed with chilled ether. 14.8 gm. of α-tetraacetylglucuronic acid methyl ester were recovered. This product contained a small quantity of the β isomer, which was separated by recrystallization.

1-Chlorotriacetylglucuronic Acid Methyl Ester—5 gm. of the β-tetraacetylglucuronic acid methyl ester were placed in a pressure bottle and 20 cc. of freshly distilled acetyl chloride were added. The mixture was cooled to −50° and saturated with anhydrous hydrogen chloride. The flask was stoppered and allowed to come to room temperature. After standing for 48 hours, the bottle was cooled, opened, and the solution transferred to a distilling flask. The acetyl chloride was removed by distillation *in vacuo*. The amorphous residue was dissolved in 5 cc. of chloroform, decolorized with a little norit, filtered, and 25 cc. of anhydrous ether were added. After cooling to 0° crystals of the halogen derivative separated from the solution. 3.8 gm. were recovered. The product was recrystallized from anhydrous ether. The substance melted at 150.5–151.5° (corrected).

Analysis—3.375 mg. substance: 2.312 mg. AgI
$C_{13}H_{17}O_9Cl$. Calculated, OCH_3 8.79; found, OCH_3 9.05
3.909 mg. substance: 1.596 mg. AgCl
$C_{13}H_{17}O_9Cl$. Calculated, Cl 10.06; found, Cl 10.10
$[\alpha]_D^{21} = (-0.35° \times 100)/(2 \times 1.050) = -16.7°$ (in chloroform)

The triacetylchloroglucuronic acid methyl ester is quite soluble in chloroform, and more difficultly soluble in ether. The substance may be recrystallized either from a mixture of chloroform and ether, or from ether itself. The compound may even be rapidly recrystallized from ethyl alcohol without serious decomposition. The substance appears to be quite stable; it may be kept for weeks in a desiccator without decomposition.

When the α form of tetraacetylglucuronic acid methyl ester is treated with acetyl chloride and hydrogen chloride, one obtains the same halogen derivative as is secured from the β isomer.

In conclusion the authors wish to express their thanks to Dr. P. A. Levene for his many helpful suggestions.

SUMMARY

The preparation of α- and β-tetraacetylglucuronic acid methyl ester and of 1-chlorotriacetylglucuronic acid methyl ester has been described.

BIBLIOGRAPHY

1. Goebel, W. F., and Babers, F. H., *J. Biol. Chem.*, **101**, 173 (1933).
2. Quick, A. J., *J. Biol. Chem.*, **74**, 331 (1927).
3. Pregl, F., Quantitative organic microanalysis, translated by Fyleman, E., Philadelphia, 2nd edition, 197 (1930).

STROPHANTHIN

XXX. THE ULTRA-VIOLET ABSORPTION SPECTRA OF TRIANHYDROSTROPHANTHIDIN AND TRIANHYDROPERIPLOGENIN DERIVATIVES

By ROBERT C. ELDERFIELD and ALEXANDRE ROTHEN

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 7, 1934)

In the carbon skeleton proposed for strophanthidin and related aglucones (Formula I) Ring I has been characterized as 6-membered. This conclusion was reached from studies of the behavior of trianhydrostrophanthidin (Formula II as given before) previously reported from this laboratory. Thus, while monoanhydrostrophanthidin and dianhydrostrophanthidin are capable of being catalytically hydrogenated, trianhydrostrophanthidin has resisted all attempts at catalytic reduction of the double bonds with the exception of the one originally present in strophanthidin.[1] This observation suggested the presence of a benzenoid linkage in trianhydrostrophanthidin. This conclusion was further strengthened by its behavior on oxidation.[2] With $KMnO_4$ an acid, $C_{20}H_{24}O_3$, is formed by oxidation of the lactone side chain to a carboxyl group presumably down to the ring in question with loss of 3 carbon atoms. With CrO_3 the molecule is attacked at another point with rupture of Ring II and the formation of a lactone directly attached to the benzenoid Ring I. These oxidative degradations are peculiar to trianhydrostrophanthidin and have not been observed in the case of the mono- or dianhydro compounds. A similar line of reasoning has been used more recently in the case of neoergosterol; that is, that its oxidation to a benzenetetracarbonic acid indicates a benzenoid structure.[3,4]

---

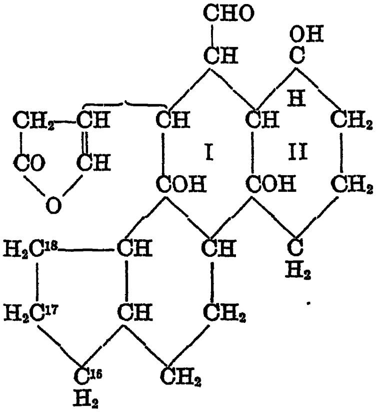
[1] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **63**, 123 (1925).

[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 805 (1927).

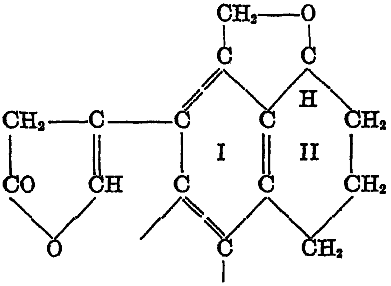
[3] Bonstedt, K., *Z. physiol. Chem.*, **185**, 165 (1929).

[4] Inhoffen, H. H., *Ann. Chem.*, **497**, 130 (1932).

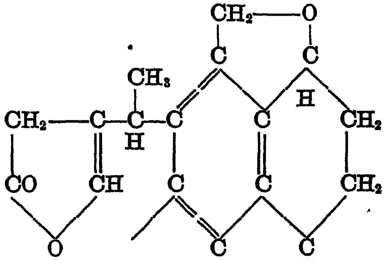
With the hope of securing additional evidence regarding the benzenoid character of Ring I in trianhydrostrophanthidin we have undertaken a study of the ultra-violet absorption spectra of a number of strophanthidin derivatives. For this purpose the series chosen was that comprising the various anhydro derivatives of dihydrostrophanthidin in order to avoid the complication of any absorption due to the double bond present in the lactone side chain



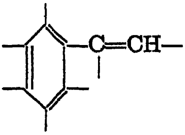
I



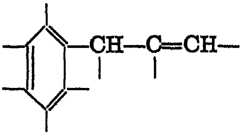
II



III



IV



V

of strophanthidin. The results of such measurements, together with the curves for certain other compounds, are shown in Figs. 1 and 2. It will be noted that in the case of monoanhydrodihydrostrophanthidin and dianhydrodihydrostrophanthidin (Fig. 1) the curves show a characteristic maximum at λ = 3030 Å. which is due to the presence of the aldehyde group. For higher frequencies the absorption increases rapidly without presenting characteristic maxima as far down in the ultra-violet as λ = 2100 Å. The curves

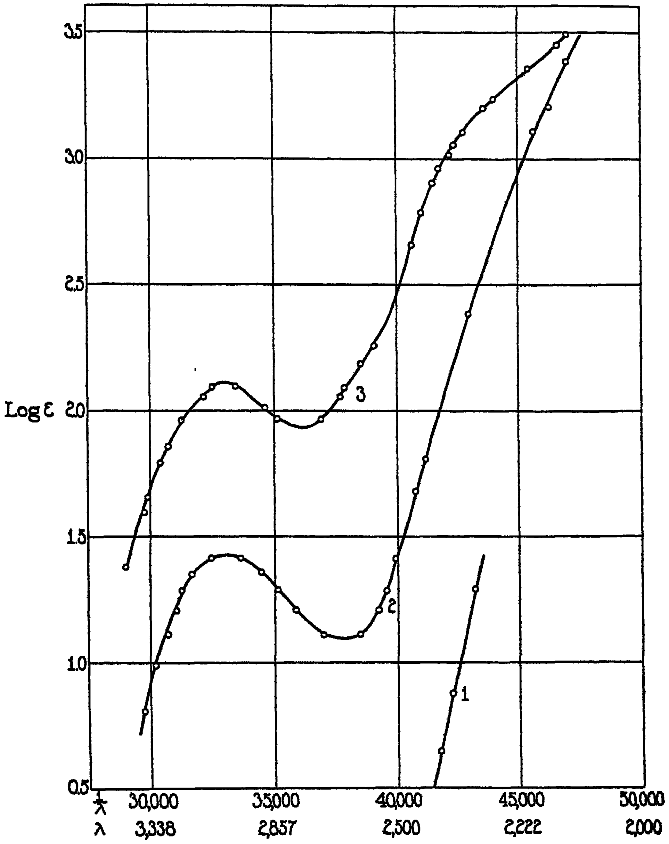


Fig. 1. Curve 1, octahydrotrianhydrostrophanthidin in dioxane; Curve 2, monoanhydrodihydrostrophanthidin in alcohol; Curve 3, dianhydrodihydrostrophanthidin in alcohol.

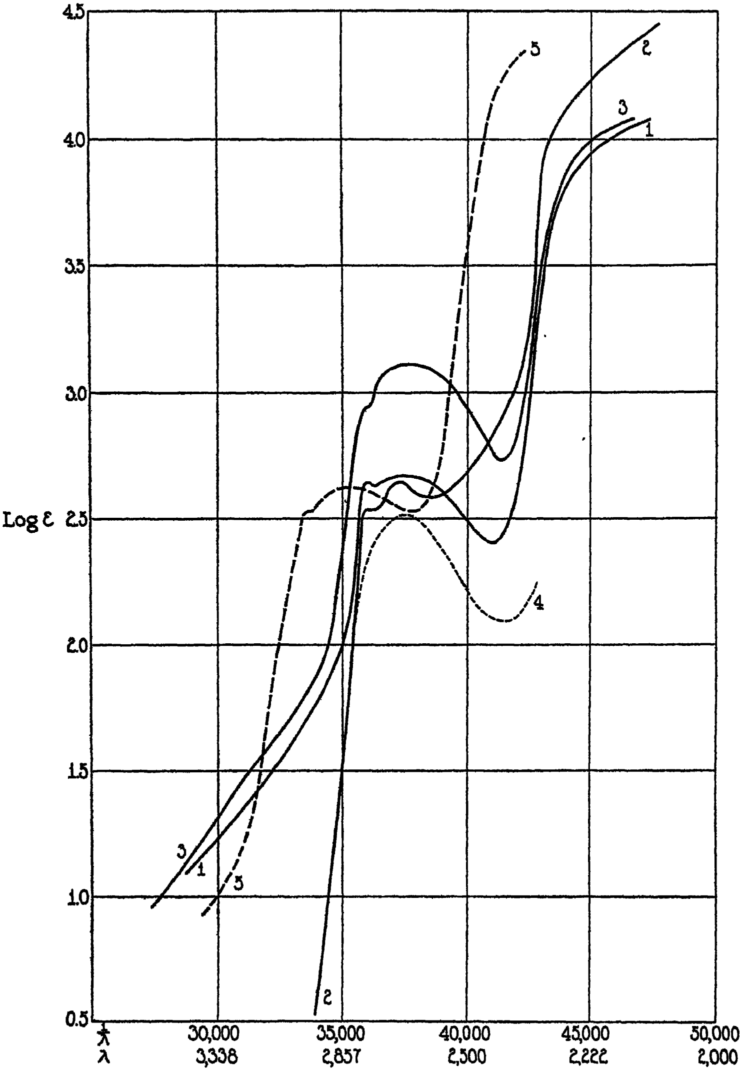


FIG. 2. Curve 1, dihydrotrianhydrostrophanthidin in alcohol; Curve 2, trianhydrostrophanthidin in alcohol; Curve 3, ester $C_{21}H_{26}O_3$ in alcohol; Curve 4, neoergosterol in ether (from Inhoffen[3]); Curve 5 trianhydroperiplogenin in dioxane.

run roughly parallel, the one for dianhydrodihydrostrophanthidin showing greater intensity, as would be expected from the presence of two double bonds. In the case of the fully saturated octahydrotrianhydrostrophanthidin the comparatively slight absorption is possibly due to the presence of the lactone carbonyl group.

In contrast to these curves, the curve for dihydrotrianhydrostrophanthidin (Fig. 2, Curve 1) shows striking differences from those in Fig. 1. A strong band is observed extending from $\lambda =$ 2790 Å. to 2500 Å., which can be resolved into two components with maxima at $\lambda = 2790$ Å. and $\lambda \simeq 2680$ Å. If we compare the intensity of the absorption of the compounds of Fig. 1 with dihydrotrianhydrostrophanthidin, it is at once obvious that, exclusive of the effect due to the aldehyde group present in the former but absent in the latter, the absorption of the former is comparatively small in the region extending from 2800 Å. to 2500 Å. It is well known that all compounds so far studied, containing one benzenoid ring, have a broad band which can be more or less resolved in that region of the spectrum. The absorption curve of dihydrotrianhydrostrophanthidin therefore indicates the presence of a benzenoid ring.

At the same time we have studied the absorption of trianhydrostrophanthidin itself and of the methyl ester of the acid, $C_{20}H_{24}O_3$, derived from trianhydrostrophanthidin by oxidation with permanganate. The similar shapes of the curves obtained at once suggest a close structural similarity of the substances of the trianhydrostrophanthidin series, two maxima being detected in the broad band, the first one having exactly the same position in all three compounds (2790 Å.). The much greater intensity observed for the band of the ester, $C_{21}H_{26}O_3$, can be easily interpreted by the presence of the carbomethoxyl group.

We have plotted on the same graph (Fig. 2) the absorption curve obtained by Inhoffen[4] in a study of the ultra-violet absorption of neoergosterol. This compound, as above noted, is assumed to possess a benzenoid ring and the position of the maximum of the band exhibited by it corresponds exactly to that found in the cases of the three trianhydrostrophanthidin derivatives. The intensity of the band is also of the same order of magnitude in the case of neoergosterol and in that of trianhydrostrophanthidin and its dihydro derivative. The same general type of curve is also shown by dihydrodesoxoestrin,[5] the maximum being displaced towards the longer wave-length as might be expected from the presence of a phenolic hydroxyl group.

In a previous communication[6] dealing with the isomeric dihydrogitoxigenins, the possibility was discussed that the unsaturated lactone side chain of the cardiac aglucones was not attached directly to Ring I. Rather the structure shown in Formula III was indicated as furnishing a possible explanation for the ease of isomerization of the dihydrogitoxigenins. In the original formula for trianhydrostrophanthidin (Formula II), the arrangement of double bonds would form a phenylethylene, or styrene system (Formula IV), whereas in the alternative formula the double bonds would comprise an allylbenzene (3-phenylpropene-(1)) system (Formula V). The recent work of Ramart-Lucas and Amagat[7] on the ultra-violet absorption spectra of various phenylated olefines would seem to offer a basis for differentiating between these two possibilities. These workers found that hydrocarbons possessing a double bond immediately adjacent to a benzene ring (phenylethylene series) irrespective of the length of the side chain gave practically identical absorption curves with a very intense maximum at $\lambda = 2450$ Å. and two subsidiary maxima at $\lambda = 2810$ Å. and 2920 Å. respectively. In contrast, compounds of the allylbenzene type or others with the double bond still further removed from the benzene ring gave absorption curves with but a single, much less intense (about 50 times less intense) band with a maximum at $\lambda = 2640$ Å. Moreover, saturation of the ethylenic double bond in a compound of the phenylethylene type to give a phenyl paraffin resulted in the production of a curve closely paralleling those of the allylbenzene type. In other words, a double bond directly adjoining a benzene ring produces a pronounced effect on the ultra-violet absorption bands due to the latter, whereas if the double bond be removed by 2 or more carbon atoms, it exerts practically no effect. In a more recent series of studies dealing with eugenol, isoeugenol, safrole, isosafrole, and related compounds, Hillmer and Schorning have confirmed this relationship between the proximity of an ethylenic double bond to a benzene ring and the ultra-violet absorption of the resulting compound,[8] thus substantiating the early work of Crymble and coworkers.[9]

---

[5] Butenandt, A., Störmer, I., and Westphal, U., *Z. physiol. Chem.*, **208**, 163 (1932).

[6] Jacobs, W. A., and Elderfield, R. C., *J. Biol. Chem.*, **100**, 671 (1933).

[7] Ramart-Lucas, and Amagat, P., *Bull. Soc. chim.*, series 4, **51**, 965 (1932).

The curve for trianhydrostrophanthidin resembles closely that for a compound of the allylbenzene type. Also, the absorption curve of dihydrotrianhydrostrophanthidin differs in no great degree from that of trianhydrostrophanthidin. If one were dealing with a phenylethylene arrangement, it would be expected that saturation of the ethylenic double bond would produce a marked effect in the absorption curve.

The formulation of the cardiac aglucones with the extended side chain in this manner is exceedingly difficult to reconcile with the sterol skeleton for these substances which is indicated by the production of Diels' hydrocarbon (methylcyclopentanophenanthrene) by selenium dehydrogenation of dianhydrouzarigenin[10] and strophanthidin.[11] An alternative to which there are serious objections would be to place the side chain on carbon atom (16), thus bringing it into conformity with the general sterol and bile acid structure. These possibilities will be discussed more fully in a forthcoming communication (Jacobs and Elderfield).

We have also investigated the absorption of trianhydroperiplogenin. This substance, while possessing three double bonds in the ring system, differs from trianhydrostrophanthidin in that all of them are comparatively easily hydrogenated.[12] Further, no definite product was obtained from trianhydroperiplogenin on oxidation with $KMnO_4$. These facts might suggest that a benzenoid structure is not present in this substance. Its absorption curve exhibits a definite broad band extending from λ = 2600 Å. to λ = 3000 Å. which undoubtedly is due to a system of conjugated double bonds. It is apparent from Fig. 2 that the curve is considerably displaced toward lower frequencies compared with the curves for trianhydrostrophanthidin and its derivatives.

---

[8] Hillmer, A., and Schorning, P., *Z. physik. Chem., Abt. A*, **167**, 407 (1933); **168**, 81 (1934).

[9] Crymble, C. R., Stewart, A. W., Wright, R., and Glendinning, W. G., *J. Chem. Soc.*, **99**, 451 (1911).

[10] Tschesche, R., and Knick, H., *Z. physiol. Chem.*, **222**, 58 (1933).

[11] Jacobs, W. A., and Elderfield, R. C., *Science*, **79**, 279 (1934).

[12] Jacobs, W. A., and Bigelow, N. M., *J. Biol. Chem.*, **101**, 697 (1933).

Thus, while the two trianhydro derivatives are undoubtedly structurally similar, the divergence of their absorption curves combined with the ease of hydrogenation of trianhydroperiplogenin indicates a somewhat different arrangement of the double bonds in the latter from that which occurs in trianhydrostrophanthidin.

At the same time, the absorption of digitaligenin (dianhydrogitoxigenin) has been studied. This compound exhibits an absorption curve totally different from any of the above substances and will be made the subject of a future communication.

EXPERIMENTAL

The absorption measurements were carried out with a Hilger sector photometer in conjunction with a Hilger model E-316 quartz spectrograph. All substances were studied in alcoholic solution with the exception of trianhydroperiplogenin and octahydrotrianhydrostrophanthidin which were dissolved in dioxane. As a check, it was ascertained that the influence of the solvent was negligible by a comparison of the absorption of trianhydrostrophanthidin in both solvents. In dioxane the intensity of the maximum of the band was but slightly depressed without displacement of its position. Results are shown in Figs. 1 and 2 where the logarithm of the molecular extinction coefficient (log ϵ) is plotted against the wave number ($1/\lambda$). The definition of molecular extinction coefficient is that adopted by the "International critical tables."[13]

---

[13] International critical tables of numerical data, physics, chemistry and technology, New York, **5**, 359 (1929).

THE OXIDATION OF PROLINE AND ALANINE BY CERTAIN TISSUES

By FREDERICK BERNHEIM and MARY L. C. BERNHEIM

(From the Departments of Physiology and Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, May 9, 1934)

It has been shown (1) that rat liver is able to oxidize proline either by the reduction of methylene blue or by the utilization of a definite quantity of oxygen. This oxidation has now been further studied in a variety of different tissues and animals, including pigeon, mouse, rat, cat, rabbit, dog, guinea pig, and human. The liver and kidney of all these are able to oxidize proline in various degrees and all are able to reduce methylene blue more rapidly in its presence. Other tissues such as spleen and muscle seem unable to oxidize this amino acid under the conditions of these experiments.

Recently Krebs (2) has shown that slices of rat kidney and liver are able to oxidize proline and also a large number of other amino acids and that this oxidation is accompanied by the liberation of ammonia. As previously reported we have found no deamination by rat liver under the conditions of our experiments, and except for the guinea pig kidney and cat liver none of the other tissues shows definite deamination. In the two exceptions we have followed the deamination at various stages of the oxygen uptake and found that deamination only occurred after a large amount of oxygen had been taken up, suggesting that the oxidation and deamination are separate processes. The failure to obtain deamination in the other tissues may therefore be due either to the absence of the deamination system or its destruction by our methods of preparing the enzyme. This may explain the apparent discrepancy between our results on the rat and those of Krebs.

The amount of oxygen taken up by the proline with the kidney and liver of different animals varies greatly. This may depend in certain cases on the quantity of enzyme present in the preparation, for by varying the concentration of tissue used different apparent end-points are obtained. On the other hand, very dilute preparations of certain tissues will utilize with the same amount of proline 4 or 5 times the amount of oxygen utilized by a concentrated preparation of another tissue. This does not necessarily mean that the processes are different in the different tissues, but merely that the oxidation in certain cases is pushed further.

Recently Aubel (3) has shown that the chopped liver of the dog will oxidize alanine to pyruvic acid. Krebs also mentions that a suspension of rat liver will do the same. We have studied the oxidation of alanine in various tissues with the same preparation that was used for the oxidation of proline. Various tissues differ in their ability to oxidize alanine but the variations do not correspond to the variations for the proline oxidation; that is, a preparation which oxidizes proline actively may have very little effect on alanine. This indicates that there are two distinct catalysts for the oxidation of these two amino acids.

TABLE I

Reduction Time of 1 Cc. of Methylene Blue (1 : 5000) by 0.5 Cc. of Tissue Alone and with 2 Mg. of Alanine, pH 7.8, at 18°

Tissue	Reduction time	
	With 2 mg. alanine	No alanine
	min.	*min.*
Rabbit kidney	35	50
Rat liver	20	30
Cat kidney	7	10
Dog liver	6	10

Unlike proline the oxidation of alanine is always accompanied by deamination, the extent of which runs parallel with the amount of oxygen taken up. Here again the concentration of tissue has an effect on the amount of oxygen taken up by the alanine and the extent of the uptake may vary from day to day in different animals. On the average about half the theoretical amount of oxygen is taken up and half the ammonia is liberated, calculated on the basis of the oxidation of alanine to pyruvic acid. But because of the variations little significance can be attached to this.

In active preparations, such as rabbit kidney or rat liver, alanine will reduce methylene blue. This is shown in Table I. This puts the catalyst for alanine with that for proline in the class of dehydrogenases. The difference in the reduction time of the kidney alone and the kidney plus alanine is not as great as in the case of proline, which corresponds to the fact that the oxidation of alanine is slower.

Table II

Effect of 0.05 M Sodium Fluoride on Oxygen Uptake of Proline and Alanine by Certain Tissues

The figures represent the final oxygen uptake, in c.mm., at 37°, of the proline or alanine and the tissue, from which was subtracted the oxygen uptake of the tissue with or without fluoride.

Tissue	2 mg. proline		2 mg. alanine	
	No fluoride	0.05 M fluoride	No fluoride	0.05 M fluoride
Rabbit liver	43	62		
	16	33	0	0
" kidney	131	202	21	138
	72	268	23	41
	102	174		
Guinea pig liver	28	112		
	82	221		
" " kidney	149	463	109	288
	118	342	86	173
	119	364		
	113	315		
Rat liver	117	161	126	138
	105	136	179	140
" kidney	29	21	146	126
	68	59		
Dog liver	136	254	78	143
" kidney	159	386		
	131	211	100	147
Mouse liver	81	132	0	16
" kidney	66	69	50	54
Pigeon liver	23	29		
	0	0	0	19
Cat liver	67	190	51	195
	141	204		
" kidney	53	104	91	120
	27	79		

The addition of various salts, particularly sodium fluoride, has a striking effect on the oxygen uptake of proline and alanine in certain tissues, although the effect is greater in the case of the former. Addition of 0.2 per cent sodium fluoride to guinea pig or rabbit kidney or cat liver or kidney may more than double the amount of oxygen taken up by the proline. Normally guinea pig kidney oxidizes proline slowly and gives an apparent end-point of about 100 to 150 c.mm. of oxygen for 2 mg. of proline. Addition of fluoride increases the rate and amount of the oxygen uptake so that the apparent uptake is 300 to 400 c.mm. of oxygen. It has, however, been found occasionally that tissues without fluoride

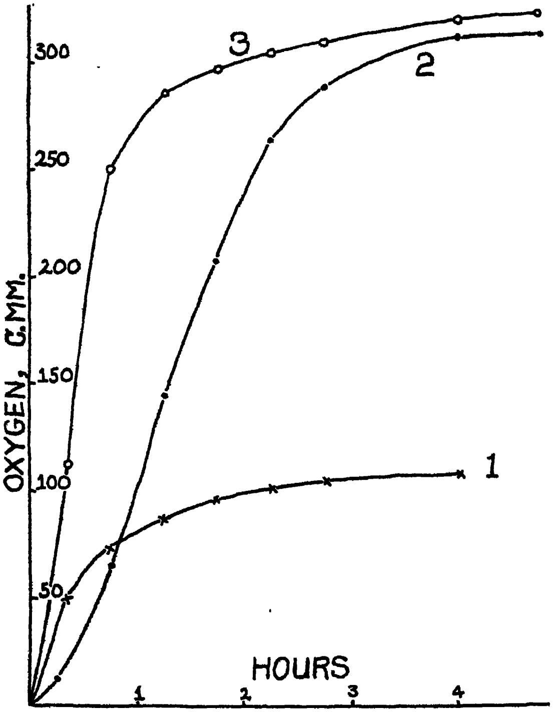


Fig. 1. The oxidation of 2 mg. of proline by guinea pig kidney, showing the effect of the addition of 0.05 M sodium fluoride and 0.05 M sodium chloride. Curve 1, proline alone; Curve 2, proline plus NaF; Curve 3, proline plus NaCl.

will oxidize proline to a greater extent than is usual. In these cases, as is to be expected, the fluoride has little or no effect.

The acceleration by fluoride can be duplicated in the case of alanine. This is shown in Table II. It is interesting that the oxygen uptake of the tissue alone is often increased by the addition of fluoride and this is sometimes accompanied by an increased deamination. Evidently fluoride may act as a general stimulant to oxidative deaminations.

Although the effect of fluoride on the kidney of the rabbit and the liver and kidney of the cat, dog, and guinea pig is marked, the effect on other tissues is somewhat less. On the kidney and liver of the rat and pigeon, for example, fluoride has little if any effect. In other cases the increase in oxygen uptake was only between 30 and 50 per cent.

TABLE III

Comparison of Oxidation and Deamination of 2 Mg. of Proline by Guinea Pig Kidney

The figures represent the percentage of the final oxygen uptake compared with the percentage of proline nitrogen present as ammonia at different times during the experiments.

		per cent	*per cent*	*per cent*	*per cent*
Experiment 1	O_2 uptake	72	100		
	NH_3-N	1.2	6.2		
" 2	O_2 uptake	54	100		
	NH_3-N	0	3.8		
" 3	O_2 uptake	33	55	87	100
	NH_3-N	1.4	1.6	2.2	3.9
" 4	O_2 uptake	29	75		
	NH_3-N	0.8	4.2		
" 5	O_2 uptake	84	100		
	NH_3-N	2.5	9.1		

In order to determine whether the acceleration was due to the specific action of the fluoride ion or was simply a salt effect, equimolar solutions of sodium and potassium chloride were added to guinea pig kidney. These salts had effects very similar to the sodium fluoride in causing acceleration of the oxygen uptake of the proline and alanine and of the kidney alone. The only difference was that whereas the fluoride effect occurred almost always, the effect of the other salts was more variable, sometimes giving large accelerations and sometimes having no effect. This variation in individuals of one species depends on a number of factors as yet undetermined. The conclusion is, however, that the fluoride effect is predominantly a salt effect. In this connection Ewig (4), Lipmann (5), and Needham (6) have shown that fluoride ion has little specific effect on the oxygen uptake of various cells. The effect of different salts on the oxygen uptake of tissues and amino acids is being further investigated. A comparison of the effects of sodium fluoride and sodium chloride on the oxidation of proline is shown in Fig. 1.

EXPERIMENTAL

As far as possible the tissues of all the animals were prepared in the same way. The animal was killed by decapitation, and the liver and kidneys were removed and chopped finely with scissors, ground in a mortar with sand, and squeezed through muslin. In order to obtain fairly comparable results 1.0 cc. of 0.05 M phosphate buffer at pH 7.8 was added for every gm. or 0.5 gm. of tissue, depending on its activity. 1 cc. of the resulting suspension was used in each Warburg vessel, 2 mg. of proline were added, and the whole made up with buffer to a volume of 2 cc. *l*-Proline and *dl*-alanine were used.

Table III gives the oxygen uptake and deamination of proline by the guinea pig kidney. The ammonia determinations were carried out under standard conditions by means of a vacuum distillation method and the ammonia determined colorimetrically after Nesslerization. The maximum ammonia nitrogen due to the 2 mg. of added proline was 0.24 mg. Control experiments showed that one-half and even one-third of this amount when added to kidney before distillation in the form of $(NH_4)_2SO_4$ could be satisfactorily recovered. Control experiments also showed that this amount of ammonia did not escape from the liquid in the Warburg vessels during the oxidation despite the pH being 7.8. Because of the small amount of ammonia to be estimated a large number of experiments were carried out and in all of them the ammonia production lagged behind the oxygen uptake.

Attempts were made to isolate the end-product of the proline oxidation. For this purpose rat liver was used because fluoride and chloride have little effect on the oxidation and because no deamination occurred. This made it possible to follow the product through various procedures by micro-Kjeldahl determinations. 100 mg. of proline were oxidized at one time with a suitable amount of liver prepared in the standard way. When the oxidation was finished, the liver was adjusted to about pH 5.0 and immersed in boiling water until the proteins had coagulated. The coagulum was filtered off, and the filtrate treated with basic lead acetate, the excess of which was removed by H_2S. The filtrate was then concentrated to about one-quarter of its original volume and treated with a small amount of charcoal and permutit to remove any free ammonia. Permutit does not absorb the end-product of the proline oxidation. From here various procedures were tried, most of them giving as the end-product a yellow and oily residue which became deeper yellow on standing but did not crystallize. This oil is water-soluble and is slightly soluble in hot methyl and ethyl alcohol but insoluble in ether and acetone. It gives no precipitate with the heavy metals but is precipitated by phosphotungstic acid and this precipitate is somewhat soluble in 90 per cent acetone. Breaking up the phosphotungstate with barium leaves the yellow oily residue apparently unchanged. In every experiment a control without proline was run. The control gave only a comparatively small precipitate with phosphotungstic acid.

Attempts to isolate or purify the enzyme responsible for the oxidation of proline have been unsuccessful. Rat liver was used, and even such mild treatments as precipitation with alcohol, acetone, or $(NH_4)_2SO_4$ almost completely inactivated it. Other ordinary procedures were also ineffective. The catalyst seems to be unusually unstable.

Through the kindness of Dr. K. A. C. Elliott of the Cancer Research Laboratory it was shown that proline could not be oxidized by various peroxidases in the presence of hydrogen peroxide.

SUMMARY

1. The oxidation of proline and alanine by broken cell suspensions of the kidney and liver of various animals has been studied.

2. The various organs show a different distribution for the cata-

lysts involved in the oxidation of proline and alanine, indicating that each has a specific catalyst.

3. 0.05 M sodium fluoride has a marked effect in accelerating and increasing the oxygen uptake of both proline and alanine by all the tissues except the kidney and liver of the mouse, rat, and pigeon.

4. This sodium fluoride effect is in part a salt effect, for similar results can be obtained, but less constantly, with equimolar solutions of sodium and potassium chloride.

5. Except in the case of the guinea pig kidney and cat liver the oxidation of proline is not accompanied by deamination and in these cases the deamination lagged behind the oxidation. The oxidation of alanine was always accompanied by deamination.

6. All tissues that oxidize proline and alanine will reduce methylene blue more rapidly in their presence.

BIBLIOGRAPHY

1. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **96**, 325 (1932).
2. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
3. Aubel, E., *Compt. rend. Soc. biol.*, **113**, 37 (1933).
4. Ewig, W., *Klin. Woch.*, **8**, 839 (1929).
5. Lipmann, F., *Biochem. Z.*, **196**, 3 (1928); **206**, 171 (1929).
6. Needham, J., *Proc. Roy. Soc. London, Series B*, **112**, 98 (1932).

THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XXXIX. THE CONSTITUTION OF TUBERCULOSTEARIC ACID[*]

By M. A. SPIELMAN[†]

(From the Department of Chemistry, Yale University, New Haven)

(Received for publication, May 15, 1934)

Anderson and his associates (1, 2) have isolated from various lipid fractions of tubercle bacilli and other acid-fast bacteria (3–7) a series of new, saturated, liquid fatty acids of unknown constitution. The simplest of these is an optically and physiologically inactive substance to which the name tuberculostearic acid has been given. The present paper is concerned with experiments which have led to a knowledge of its structure.

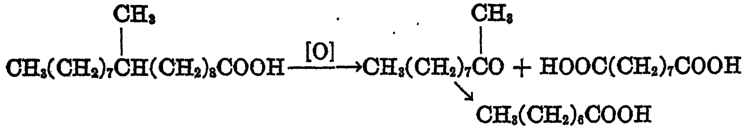
The empirical formula of the purified tuberculostearic acid was established as $C_{19}H_{38}O_2$ on the basis of many determinations of the molecular weight by titration and by analyses of the free acid and several functional derivatives, including the silver salt. In earlier papers from this laboratory, the formula $C_{18}H_{36}O_2$ was favored, although analytical values were usually too high. The reason lay in a reluctance to attribute to a natural fatty acid a formula having an odd number of carbon atoms. In the absence of adequate criteria of purity, there was never assurance that a homogeneous substance was at hand. In the case of a specimen isolated from *Bacillus lepræ*, Anderson and Uyei (6) have pointed out the better agreement with the C_{19} formula.

The problem of structure was approached through drastic oxidation of tuberculostearic acid with chromic acid in glacial acetic acid solution. Primary oxidation products seemed to be attacked as readily as the substance itself; hence, they were obtained in exceedingly small yield. Nevertheless, two relevant products, namely methyl *n*-octyl ketone and azelaic acid, were isolated and definitely characterized. Caprylic acid, formed largely as a secondary product, could not be isolated in a pure state, although evidence of its presence was satisfactorily shown. Tuberculostearic acid is therefore believed to consist largely, if not entirely, of 10-methylstearic acid, and the degradation products are formed as shown in the following reaction.

---

[*] The present report is a part of a cooperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

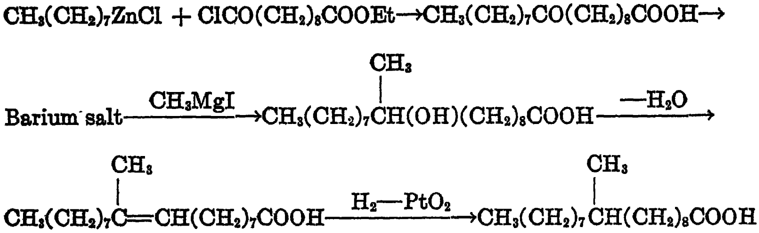
[†] National Research Council Fellow in Chemistry.



$$CH_3(CH_2)_7\overset{\overset{\displaystyle CH_3}{|}}{C}H(CH_2)_8COOH \xrightarrow{[O]} CH_3(CH_2)_7\overset{\overset{\displaystyle CH_3}{|}}{C}O + HOOC(CH_2)_7COOH$$

$$\searrow CH_3(CH_2)_6COOH$$

For purposes of comparison, a specimen of *dl*-10-methylstearic acid was synthesized by means of the following reactions.



$$CH_3(CH_2)_7ZnCl + ClCO(CH_2)_8COOEt \rightarrow CH_3(CH_2)_7CO(CH_2)_8COOH \rightarrow$$

$$\text{Barium salt} \xrightarrow{CH_3MgI} CH_3(CH_2)_7\overset{\overset{\displaystyle CH_3}{|}}{C}H(OH)(CH_2)_8COOH \xrightarrow{-H_2O}$$

$$CH_3(CH_2)_7\overset{\overset{\displaystyle CH_3}{|}}{C}{=}CH(CH_2)_7COOH \xrightarrow{H_2-PtO_2} CH_3(CH_2)_7\overset{\overset{\displaystyle CH_3}{|}}{C}H(CH_2)_8COOH$$

The first step, the synthesis of 10-ketostearic acid, is essentially that of Fordyce and Johnson (8), although by a slight modification of their technique the yield was trebled. The reaction of a Grignard reagent with an oxo-acid derivative, as in the third step, is a procedure much employed by Adams and coworkers (9). These investigators, however, used the esters, while in the present work it was found that by employing the barium salt, and carrying out the reaction in a two-phase system, much better results were obtained. The balance of the synthesis presents nothing new.

Synthetic *dl*-10-methylstearic acid resembles tuberculostearic acid very closely, but identity cannot be claimed. Melting points differ by 10°, and the solid derivatives which were prepared differ in crystalline form. On the other hand, these derivatives possess identical melting points, and mixed melting points are not depressed. Furthermore, the two acids form the same peculiar lead salt which is easily soluble in ether and sparingly so in 95 per cent alcohol. Densities and refractive indices are practically identical. The data are compared in Table I.

The differences between the natural and synthetic acids can be attributed to either or both of two things. The most obvious explanation is that the fraction designated as tuberculostearic acid still contains enough of an isomeric substance to depress its melting point. Methods of purification and criteria of purity are so poorly developed in the field of liquid fatty acids that the presence of an impurity such, for instance, as 9-methylstearic acid, might easily escape detection. What seems to the author to be a more reasonable explanation is that the differences are connected with the presence of an asymmetric carbon atom. The fact that tuberculostearic acid shows no optical activity does not prove its racemic nature. If the data of Levene, Rothen, and Marker (10), dealing with optical properties of methyl derivatives of fatty acids, are extended to such a substance as that under consideration, the absence of measurable rotation is not surprising. The closest approach is δ-methyldecylic acid whose molecular rotation is only 0.6. Furthermore, natural racemates are rare, and racemization during isolation is unlikely because concurrent higher products such as phthioic acid retain their activity. A similar anomaly has been encountered by Chibnall and associates (11) who isolated an inactive 10-nonacosanol from plant waxes, but found that it differed slightly from their synthetic product in its melting point. Later, by the preparation of an acid phthalate, they were able to show that it was not racemic. Racemates, such as camphoric acid, which melt higher than either antipode, are too numerous to require citation. Whatever the explanation, the matter can

TABLE I

Comparison of Tuberculostearic and dl-10-Methylstearic Acids

	M.p.	n_D^{25}	d_4^{25}	Me ester		Amide m.p.	Tribromoanilide m.p.
				n_D^{25}	d_4^{25}		
	°C.					°C.	°C.
Tuberculostearic.........	10–11	1.4512	0.8771	1.4438	0.8614	76–77	93–94
10-Methylstearic.........	20–21	1.4512	0.8769	1.4438	0.8621	76–77	93–94

be cleared up when a method is evolved for the resolution of our synthetic acid.

EXPERIMENTAL

Purification and Preparation of Derivatives

Methyl Tuberculostearate—The starting material consisted of various samples isolated from tubercle bacilli, Strain H-37, which had accumulated in this laboratory during the past several years. All of it had been distilled three to seven times at pressures of about 0.004 mm. It was fractionated three times more at 3.5 mm. in a modified Claisen flask having an 8 cm. indented column; the part collected boiled at 158–159.5°. The ester was saponified and the free acid was collected. It still appeared to contain a little stearic acid which had escaped the original lead soap-ether separation. This was removed as much as possible by freezing out in methanol (15 cc. of solvent per 10 gm. of acid). Further purification was along the lines of the Twitchell procedure (12); 17 gm. of acid were dissolved in 400 cc. of hot 95 per cent alcohol and exactly neutralized with alcoholic potash. 12 gm. of lead acetate in 200 cc. of hot alcohol were added. After standing overnight at 15° the gummy precipitate was separated and taken up in 30 cc. of ether. On standing, a small amount of lead stearate came out and was removed by filtration through norit. The ether-soluble lead salts were decomposed and 9.4 gm. of acid were collected. It melted at 10°. The alcoholic mother liquors were worked up for their acid content; this was dissolved in 200 cc. of alcohol, treated with 4 gm. of lead acetate, and from the precipitated lead salt an additional 2.8 gm. of acid melting at 10° were isolated. The alcohol-soluble lead salts were set aside, and the two main fractions of liquid acid were methylated and distilled. Practically all came over at 143–144.5° at 1.5 mm. $n_{D}^{25} = 1.4438$, $d_{4}^{25} = 0.8614$.

Saponification—0.3757 gm. required 11.97 cc. 0.1 N KOH
$C_{20}H_{40}O_2$. Mol. wt. calculated, 312.3; found, 313.9
Analysis—0.01415 gm. substance: 0.01602 gm. H_2O and 0.03990 gm. CO_2
$C_{20}H_{40}O_2$. Calculated. C 76.85, H 12.90
Found. " 76.90, " 12.67

Tuberculostearic Acid—The ester was saponified and the free acid was collected after a preliminary extraction of the saponification mixture to insure the absence of neutral material. It was a nearly colorless oil which solidified readily on cooling and melted in a capillary at 10–11°. $n_D^{25} = 1.4512$, $d_4^{25} = 0.8771$.

Neutralization—0.2191 gm. required 7.35 cc. 0.1 N KOH
$C_{19}H_{38}O_2$. Mol. wt. calculated, 298.3; found, 298.0
Analysis—0.01821 gm. substance: 0.02109 gm. H_2O and 0.05119 gm. CO_2
$C_{19}H_{38}O_2$. Calculated. C 76.43, H 12.84
Found. " 76.67, " 12.91

In order to be sure that the analytical values were not due to a fortuitous mixture of acids, a sample was precipitated with lead acetate in three successive fractions which were titrated separately. Figures for the molecular weight were 298.5, 299.3, and 297.0. Two other independent samples gave 299.1 and 297.0.

The silver salt was prepared according to the directions of Anderson and Chargaff (2).

Analysis—0.2757 gm. substance gave on ignition 0.0740 gm. Ag
$C_{19}H_{37}O_2Ag$ (404.9). Ag calculated, 26.65; found, 26.84

Tuberculostearamide—0.5 gm. each of the acid and phosphorus pentachloride were heated on a water bath until the reaction was complete. The mixture was poured into cold ammonia and the product was formed in nearly quantitative yield. It was extremely soluble in all organic solvents but was crystallized successfully from dilute methanol. Needles, melting point 76–77°.

Analysis—0.01473 gm. substance: 0.01744 gm. H_2O and 0.04154 gm. CO_2
$C_{19}H_{39}NO$ (297). Calculated. C 76.77, H 13.13
Found. " 76.91, " 13.25

Tuberculostearo-2,4,6-Tribromoanilide—The directions were those of Robertson (13). The product was decolorized with norit in methanol and crystallized from the same solvent. Yield 50 per cent; needles, melting point 93–94°.

Analysis—0.01830 gm. substance: 0.01092 gm. H_2O and 0.03300 gm. CO_2
$C_{25}H_{40}NOBr_3$ (610). Calculated. C 49.18, H 6.67
Found. " 49.18, " 6.61

Oxidation of Tuberculostearic Acid

Many sets of conditions were tried, but each refinement seemed to lessen the yield of degradation products, and finally the following procedure was adopted. 2 gm. of acid and 50 cc. of glacial acetic acid, freshly distilled from chromic acid, were placed in a 250 cc. Erlenmeyer flask and cautiously heated on a hot plate while 4 gm. of chromic acid were added in four portions with shaking. Whenever the evolution of carbon dioxide became vigorous, the flask was momentarily transferred to a vessel of cold water. Heating and cooling were so adjusted that the reaction required about 10 minutes. The mixture was diluted with water, a few drops of concentrated sulfuric acid were added, and a current of steam was passed through until 300 cc. of distillate had been collected. A few oily droplets floated on the distillate and a pleasant mint-like odor was apparent. There was no odor of lower fatty acids and a test portion gave no indication of water-soluble ketones. The ether extract of the distillate was washed free of acetic acid with water and separated into neutral and acid fractions with dilute alkali.

The neutral volatile portion after cautious evaporation of the ether left a very small amount of colorless, fragrant oil. A minute droplet transferred to a drop of potassium hypoiodite under a microscope gave the characteristic yellow crystals of iodoform, thus indicating that the unknown was a methyl ketone. It was taken up in 5 cc. of alcohol and warmed with 50 mg. each of semicarbazide hydrochloride and sodium acetate. After 5 minutes it was diluted to turbidity and allowed to crystallize. The precipitate weighed 30 mg. and after several crystallizations from dilute alcohol the melting point became constant at 122–123° which is correct for the semicarbazone of methyl *n*-octyl ketone (14). A specimen was synthesized and the mixed melting point was not depressed.

Analysis—0.01412 gm. substance: 0.01366 gm. H_2O and 0.03190 gm. CO_2

$C_{11}H_{23}N_3O$ (213.12). Calculated. C 61.97, H 11.05
Found. " 61.62, " 10.82

The steam-volatile acid was converted to the amide (90 mg.) or to the *p*-bromophenacyl ester (80 to 160 mg.) by the usual methods. Neither could be satisfactorily purified. The amide melted at 100–103°, whereas caprylamide should melt at 105° (13).

Analysis—0.01456 gm. substance: 0.01542 gm. H_2O and 0.03613 gm. CO_2

$C_8H_{17}NO$ (143). Calculated. C 67.13, H 11.89
Found. " 67.68, " 11.85

The *p*-bromophenacyl ester melted at 60–62°; the same derivative of caprylic acid should melt at 65.5° (15).

Analysis—0.01913 gm. substance: 0.01090 gm. H_2O and 0.03985 gm. CO_2
$C_{16}H_{21}O_2Br$ (341.1). Calculated. C 56.30, H 6.16
Found. " 56.68, " 6.37

The material remaining after the steam distillation described above was extracted with ether, washed with water until the washings were colorless, and separated into neutral and acid fractions. The neutral part yielded nothing of interest. The acid fraction was extracted with four 10 cc. portions of boiling water by decantation through a wet filter. The extract was concentrated to 2 cc. and allowed to crystallize. A colorless solid was isolated in yields of 30 to 70 mg. After sublimation *in vacuo* and crystallization from water and from benzene, it melted at 105° and its melting point was not changed when mixed with pure azelaic acid.

Titration—0.0118 gm. required 1.23 cc. 0.1 N KOH
$C_9H_{16}O_4$. Mol. wt. calculated, 94.1; found, 96.0
Analysis—0.01152 gm. substance: 0.0087 gm. H_2O and 0.0241 gm. CO_2
$C_9H_{16}O_4$. Calculated. C 56.84, H 8.43
Found. " 57.06, " 8.45

Synthesis of dl-10-Methylstearic Acid

10-Ketostearic Acid—A 500 cc. 3-neck flask was fitted with an inlet tube for hydrogen in addition to the usual equipment for the Grignard reaction. A reagent was prepared from 15 gm. of *n*-octyl bromide, 1.87 gm. of magnesium, and 100 cc. of ether in an atmosphere of pure, dry hydrogen. It was cooled in an ice bath while a solution of 10.6 gm. of freshly fused zinc chloride in 16 cc. of ether was added slowly with vigorous stirring. A white solid began to separate at once. A downward condenser was connected and most of the ether was boiled off on a water bath; 200 cc. of sodium-dried benzene were added, and heating was continued until the boiling point reached 79°. The volume was brought to 200 cc. with more benzene, and while the mixture was boiled and stirred, 19.3 gm. of ω-carbethoxynonoyl chloride (8) were added in an equal volume of benzene. A gummy solid began to form, and toward the last of the reaction stirring became largely ineffectual. The product was decomposed with dilute hydrochloric acid and washed with ammonium nitrate solution. The benzene was removed and the residue was saponified and freed of neutral material by extraction of the saponification mixture with ether. The acids were suspended in 800 cc. of water, made strongly alkaline with ammonia, filtered, and washed thoroughly with water, dilute hydrochloric acid, and again with water. The product was purified by crystallization from methanol, and the yield of 10-ketostearic acid melting at 80–82° was 40 per cent. A larger run yielded 34 per cent.

Methyl 10-Methylstearate—10-Ketostearic acid was converted to the barium salt by neutralization of an alcoholic solution with barium hydroxide. 21 gm. of the thoroughly dried chalky powder were suspended in 250 cc. of ether in a 3-neck flask and stirred until disintegration was complete and a smooth cream resulted. Through the dropping funnel was added a Grignard solution prepared from 18.2 gm. of methyl iodide and 3.3 gm. of magnesium in 50 cc. of ether. The mixture was stirred for 5 hours, during the last 2 of which it was boiled. It was decomposed and the product was separated into neutral and acid fractions in the customary manner. The neutral part weighed 8.8 gm. and was not further examined. The acids were dissolved in 25 cc. of 95 per cent methanol, and cooling in ice-salt caused the separation of 0.4 gm. of unchanged 10-ketostearic acid. The crude, oily 10-methyl-10-hydroxystearic acid weighed 9.5 gm., representing a yield of 52 per cent. (By using less Grignard reagent the net yield was brought up to 66 per cent but more unchanged acid was recovered.) Dehydration proceeded smoothly on heating to 175° with a trace of iodine. The mixed unsaturated acids were methylated and distilled; 7.8 gm. were collected at 158–160° (3.5 mm.). This was reduced in methanol with the usual platinum oxide catalyst, and the product, methyl 10-methylstearate, was distilled; boiling point 154–156° at 3 mm. $n_D^{25} = 1.4438$, $d_4^{25} = 0.8621$.

Analysis—0.1346 gm. substance: 0.01534 gm. H_2O and 0.03769 gm. CO_2

$C_{20}H_{40}O_2$ (312.3). Calculated. C 76.85, H 12.90

Found. " 76.37, " 12.76

dl-10-Methylstearic Acid—The free acid was obtained on saponification of the ester. It melted at 20–21° and when mixed with tuberculostearic acid the melting point was intermediate but less sharp than that of either component. $n_D^{25} = 1.4512$, $d_4^{25} = 0.8769$.

Titration—0.2609 gm. required 8.82 cc. 0.1 N KOH
$C_{19}H_{38}O_2$. Mol. wt. calculated, 298.3; found, 296.0
Analysis—0.01350 gm. substance: 0.01535 gm. H_2O and 0.03762 gm. CO_2
$C_{19}H_{38}O_2$. Calculated. C 76.43, H 12.84
Found. " 76.00, " 12.76

10-Methylstearamide—Platelets; melting point and mixed melting point 76–77°.

Analysis—0.01482 gm. substance: 0.01731 gm. H_2O and 0.04144 gm. CO_2
$C_{29}H_{39}NO$ (297). Calculated. C 76.77, H 13.13
Found. " 76.28, " 13.07

10-Methylstearo-2,4,6-Tribromoanilide—Needles, slightly stouter than the natural product; melting point and mixed melting point 93–94°.

Analysis—0.01253 gm. substance: 0.00755 gm. H_2O and 0.02256 gm. CO_2
$C_{25}H_{40}NOBr_3$ (610). Calculated. C 49.18, H 6.67
Found. " 49.10, " 6.67

SUMMARY

Tuberculostearic acid has been purified and found to possess the empirical formula $C_{19}H_{38}O_2$. Drastic oxidation yields azelaic acid and methyl *n*-octyl ketone. These products point to the constitutional formula of 10-methylstearic acid. A sample of the racemic substance has been synthesized for comparison.

As indicated by the title, this work constitutes a part of the general investigation of bacterial lipids carried on in this laboratory by R. J. Anderson whose generosity and cooperation are gratefully acknowledged.

BIBLIOGRAPHY

1. Anderson, R. J., *J. Biol. Chem.*, **74**, 537 (1927); **83**, 169 (1929).
2. Anderson, R. J., and Chargaff, E., *J. Biol. Chem.*, **85**, 77 (1929–30); *Z. physiol. Chem.*, **191**, 157 (1930).
3. Anderson, R. J., and Roberts, E. G., *J. Biol. Chem.*, **85**, 519 (1929–30); **89**, 599 (1930).
4. Pangborn, M. C., and Anderson, R. J., *J. Biol. Chem.*, **94**, 465 (1931–32).

5. Burt, M. L., and Anderson, R. J., *J. Biol. Chem.*, **94**, 451 (1931–32).
6. Anderson, R. J., and Uyei, N., *J. Biol. Chem.*, **97**, 617 (1932).
7. Pangborn, M. C., Chargaff, E., and Anderson, R. J., *J. Biol. Chem.*, **98**, 43 (1932).
8. Fordyce, C. R., and Johnson, J. R., *J. Am. Chem. Soc.*, **55**, 3368 (1933). Compare also Späth, E., and Darling, S. F., *Ber. chem. Ges.*, **63**, 737 (1930).
9. Noller, C. R., and Adams, R., *J. Am. Chem. Soc.*, **48**, 1074 (1926). Tomencko, C. G., and Adams, R., *J. Am. Chem. Soc.*, **49**, 522 (1927).
10. Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Physics*, **1**, 662 (1933).
11. Chibnall, A. C., Piper, S. H., Pollard, A., Smith, J. A. B., and Williams, E. F., *Biochem. J.*, **25**, 2097 (1931).
12. Twitchell, E., *J. Ind. and Eng. Chem.*, **13**, 807 (1921).
13. Robertson, P. W., *J. Chem. Soc.*, **115**, 1210 (1919).
14. Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 57 (1911). St. Pfau, A., *Helv. chim. acta*, **15**, 1267 (1932).
15. Judefind, W. L., and Reid, E. E., *J. Am. Chem. Soc.*, **42**, 1043 (1919).

A STUDY OF THE ABSORPTION SPECTRA OF SOME CAROTENOID PIGMENTS AT LIQUID AIR TEMPERATURES AND ITS APPLICATIONS TO THE CAROTENOID PIGMENTS OF COW-PEA LEAVES (VIGNA SINENSIS)

BY GUIDO E. HILBERT AND EUGENE F. JANSEN

(*From the Division of Fertilizer Investigation, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*)

(Received for publication, May 15, 1934)

In general, the absorption spectra of organic substances at very low temperatures are characterized by a marked sharpening and often an increase in the gross structure of the bands. In contrast to the aliphatic type of compound, the individual bands of the simpler aromatic compounds (1) at liquid air temperatures are usually resolved into several sharp, narrow bands. Conant and Kamerling (2) found that the absorption spectra of the porphyrins behave in a manner similar to that of the benzenoid class of compounds, and they concluded that this phenomenon is unique with substances containing rigid planar rings in which there is a complete conjugation of the double bonds.

With the exception of a contemporary note by Bowden, Morris, and Snow (3) on the absorption spectrum of "carotene" at "low temperatures," apparently no other work has been done on a study of the absorption spectra of the carotenoid pigments at low temperatures. Since the conjugated double bonds in these pigments do not comprise a closed system, the resolution of the individual bands into several narrower ones at low temperatures was not anticipated. However, in addition to the expected displacement and sharpening of the bands at low temperatures, it was hoped that a certain amount of additional structure would possibly develop as this would enhance considerably the spectroscopic method as an aid in the identification of the pigments, for which there is as yet no absolute method.

Method for Obtaining Absorption Spectra at Liquid Air Temperatures

The pigments studied were α-carotene, β-carotene, carrot "carotene," "leaf xanthophyll," lycopene, and a mixture of β-carotene and leaf xanthophyll. Leaf xanthophyll from stinging nettle was supplied by Dr. Frank M. Schertz, lycopene by Dr. M. B. Matlack and Dr. C. E. Sando (4), and specimens of α-carotene by Professor R. Kuhn and by Dr. M. B. Matlack, who prepared it after the method of Karrer and Walker (5).[1] The leaf xanthophyll was recrystallized several times from methanol, and β-carotene, prepared from stinging nettle by the usual procedure (6), from petroleum ether. Crystalline carrot carotene, obtained from the more soluble fractions of a benzene-methanol solution, in which there is a higher concentration of α-carotene, was used.

The method for working at low temperatures is essentially that outlined by Conant and Crawford (7), the most important feature of which is the use of a mixture of ether and alcohol as solvent for the pigments, since this on cooling forms a transparent gel instead of a crystalline solid. The technique we have used is simple and sufficiently accurate for this type of work; a similar method with more refinements is that of Arnold and Kistiakowsky (1). An Adam Hilger medium quartz spectrograph No. E-3 was used. The light source was a 60 watt tungsten lamp. The absorption cell was made of Pyrex glass and was about 25 mm. wide, about 8 cm. high, and had a depth of light path of 6.4 mm. At the center of the top of the cell there was attached a glass tube about 12 mm. in diameter and about 11 cm. long.

The solutions, from which the absorption spectra were obtained, were prepared by dissolving 1 mg. of the pigment, which was weighed out rapidly on a microbalance, in 50 cc. of anhydrous ether to which 50 cc. of absolute methanol were then added. The cell filled with the solution was suspended by means of a slit rubber stopper, which was fitted to a 1 pint unsilvered Pyrex Dewar flask. The stoppering of the Dewar flask in this fashion was necessary in order to minimize frosting. After the cell had been adjusted so that the level of the solution in the cell was somewhat below that of the liquid air, which had been clarified by filtering through filter paper, the solution was allowed to stand for about

---

[1] These two specimens of α-carotene were spectroscopically identical.

3 minutes, when it had solidified to a clear gel and attained a temperature approximating that of liquid air. A portion of the cell was then exposed above the liquid air so that light could be transmitted through the solution onto the slit of the spectrograph. The cell was exposed for various lengths of time, although it was impractical to expose it for longer than 2 minutes. If the cell was again immersed in the liquid air bath, the gel cracked and became unsuitable for further work. This cracking could only be avoided by rewarming the cell until the gel had liquefied before recooling.

In addition to Seed 23 photographic plates which were employed in the main, a few panchromatic plates were used to show the absence of absorption bands in the region in which the former are not sensitive. For purposes of comparison the spectra of a helium discharge tube and of the solution at room temperature together with the spectra of the cooled solution were always photographed on the same plate. Since liquid air absorbs light in the visible region of the spectrum, the results obtained by transmitting light through the liquid air as well as the solution were found to be unsatisfactory.

DISCUSSION

The comparative study of the absorption spectra of α- and β-carotene, lycopene, and leaf xanthophyll taken at room and liquid air temperatures showed in general the following important changes. At low temperatures (*a*) more bands developed in the region of the near ultra-violet, (*b*) a certain amount of structure in the form of lines or bands was produced, and (*c*) as expected, because of the decrease in the heat motion of the particular portion of the molecule responsible for the absorption, the bands were sharpened considerably. They were also shifted on an average of about 150 Å. towards longer wave-lengths.

The results shown in Table I were obtained with alcohol-ether solutions containing 0.001 per cent pigment. This concentration was experimentally found to give the best results at low temperatures; at room temperature conditions a stronger solution is desirable. It is interesting to note that at low temperatures the absorption spectrum of recrystallized carotene from carrots, which contains about 10 to 15 per cent of α-carotene, is quite similar to the spectrum of β-carotene; the centers of the absorption bands lie at 4980, 4635, and 4355 Å., and thus have been shifted about 20 Å. towards shorter wave-lengths. A similar result was obtained with a solution of pigment containing 83 per cent β-carotene and 17 per cent leaf xanthophyll. This confirms the recent findings of Smith and Milner (8) who noted that the absorption maxima were progressively shifted towards shorter wave-lengths with increasing concentration of α-carotene in a mixture of α- and β-carotene. One may thus conclude that a by-product whose absorption bands overlap that of the principal substance will produce an apparent shift in the bands of the principal substance in the direction of the absorbing bands of the by-product.

TABLE I

Positions of Centers of the Absorption Bands in Ångstrom Units

α-Carotene		β-Carotene		Leaf xanthophyll		Lycopene	
Room temperature	Liquid air temperature	Room temperature	Liquid air temperature	Room temperature	Liquid air temperature	Room temperature	Liquid air temperature
4770	4910	4815	5000	4760	4900	5040	5205
4435	4590	4490	4670	4430	4580		5030
4190	4310	4230	4375	4190	4300	4710	4845
	4070		4125		4060	4425	4550
							4480
							4290

As this shift in the case of carrot carotene is small and because of the wide use in the past of the spectroscopic method as an aid in the identification of the pigments, it is not surprising that the mixed nature of carrot carotene remained unknown for such a long time. It should perhaps be stressed that the spectroscopic method as used in this field is of little use in the detection of moderate amounts of impurities.

The similarity in the absorption spectra of α-carotene and leaf xanthophyll has been commented on in the past. At liquid air temperatures the likeness is even more striking. The centers of the three main bands, as well as the one developed at low temperatures, differ by only 10 Å.; the centers of the bands of α-carotene lie closer to the longer wave-lengths and thus between those

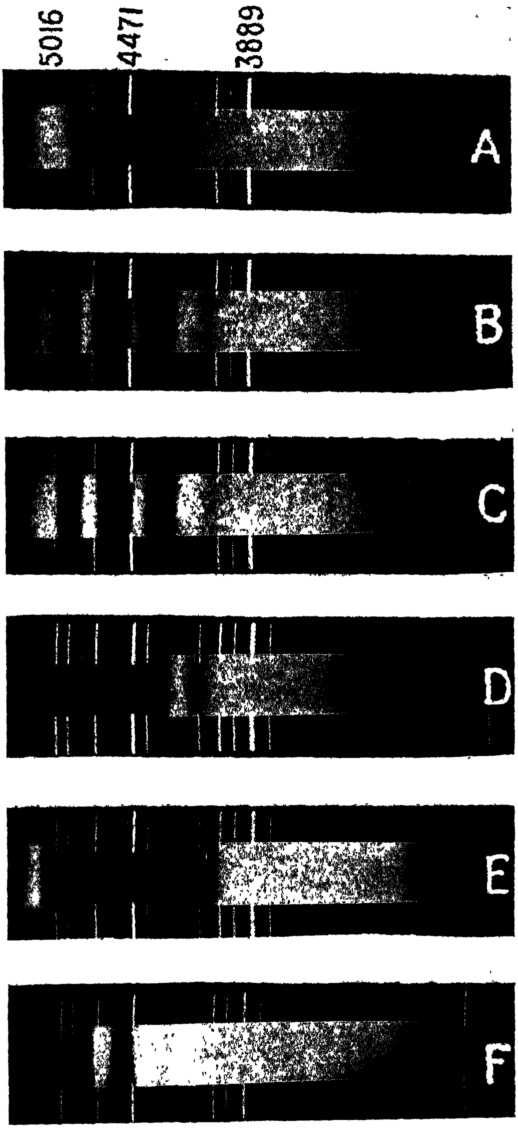


FIG. 1. Spectrograms of some carotenoid pigments; *A* and *B*, α-carotene; *C*, leaf xanthophyll; *D* and *E*, β-carotene; *F*, lycopene. *A* was obtained at room temperature and the others at liquid air temperatures. The concentration of *E* was 0.0029 per cent in 50:50 methyl alcohol-ether and was increased in order to bring out the band at 4290 Å. more clearly.

of leaf xanthophyll and β-carotene. Moreover, each spectrum has a faint transmission band in the center of the band lying at 4900 to 4910 Å., as well as a fifth absorption region whose center is approximately at 3810 Å. Attempts to intensify these features by varying the concentration were without success. This close similarity in the absorption spectra of α-carotene and leaf xanthophyll suggests that there is perhaps a closer structural relationship between these two pigments than between the latter and β-carotene. At low temperatures a fifth band whose center lies at about 3880 Å. is visible in the spectrum of β-carotene. The largest change was noted in the absorption spectrum of lycopene. In this case considerable structure is developed at low temperatures, as is illustrated in *F*, Fig. 1. A number of details could not be reproduced in this photograph, as for instance a faint transmission line in the center of the 5205 Å. band and a band at 4020 Å. The 4290 Å. band consisted of a number of transmission and absorption bands which were too diffuse to measure.

Bowden, Morris, and Snow (3) have determined the absorption spectrum of an ethanol solution of carotene at "low temperatures." In the main our results confirm theirs. They noted at the lower temperatures that the bands were sharpened and appreciably shifted towards longer wave-lengths, and that a fourth band also made its appearance. The absorption bands of other pigments were apparently not determined and a description of their method was not reported.

Carotenoid Pigments of Cow-Pea Leaves (Vigna sinensis)

The isolation of zeaxanthene ester from *Physalis* sp. (9, 10) has suggested the possibility of the occurrence of xanthophyll as an ester in certain of the green leaves. Willstätter and Stoll (11), however, were able to isolate some xanthophyll from *Urtica*, L., even in the absence of the usual alkaline hydrolysis. This result has since been supported by the work of Karrer *et al.* (12) who used a somewhat different procedure. The most comprehensive study has been that of Kuhn and Brockmann (13); they found in the several species investigated that the major portion of the xanthophyll is in the unesterified form. Because of these and other results there has been a tendency to assume that the predominant carotenoid pigments in green leaves are β-carotene and xanthophyll. Since Mackinney and Milner (14) have shown that carrot leaves contain an appreciable amount of α-carotene, and Yamamoto and Muraoka (15) have shown that the carotene in tea leaves is practically all of the α type, it is obvious that such an assumption does not hold and that the carotenoid pigments occurring in the green leaves of species not yet investigated may well show other differences.

We were led to investigate the carotenoid pigments of *Vigna sinensis* through some observations of Dr. Frank M. Schertz. He called our attention to the fact that the carotene extract obtained from this species, in contrast to the golden yellow color of that obtained from *Urtica dioica*, L., has a decided brownish cast which perhaps suggests the presence of a different type of pigment. Moreover, as the lipid content of *Vigna sinensis* is high and the environment thus favorable for the production of xanthophyll esters, the nature of the xanthophyll was also investigated.

The method used for determining whether or not xanthophyll is present as an ester was essentially similar to those used in the past. The dried leaves were extracted with petroleum ether, since the xanthophyll esters are soluble in this solvent whereas xanthophyll is not. This extract after some manipulation was treated with alcoholic potash and the xanthophyll and carotene worked up in the usual manner. Xanthophyll was found to be present only in traces and from this it may be inferred that if xanthophyll is present as an ester it is so only in negligible amounts. The major portion of the carotene, which is particularly difficult to isolate from this species as might be expected because of the large amount of fatty material, was shown spectroscopically to be the β variety. The brownish color of the crude carotene extract was due to chlorophyll degradation products, probably produced by a decomposition resulting from the slow rate of drying which could not be avoided because of the thick, waxy coating on the leaves.

EXPERIMENTAL

Cow-pea leaves were dried in a rapid stream of air at 40° for 72 hours. When dry, the leaves were ground in a ball mill to a fine meal. 400 gm. of this were treated with 1 kilo of petroleum ether (b. p. 35–50°) and allowed to stand overnight before filtering by suction and washing with 500 cc. of the solvent.

Leaf Xanthophyll—The greenish colored filtrate had a pronounced yellowish tint and was washed successively with 150 cc. portions of 80, 85, 85, and 90 per cent methanol in order to remove free xanthophyll. The petroleum ether fraction was dried over sodium sulfate and treated with a solution of 2 gm. of sodium in 40 cc. of 95 per cent ethanol. During this operation, the solution lost its fluorescence and, as no xanthophyll separated, which might be expected were it present as an ester, it was shaken with portions of 10 per cent methyl alcoholic potash until the green color was removed and then with 85 per cent methanol. The alcoholic and alcoholic potash extractions were combined and, after the addition of some water, thoroughly extracted with ether. After drying the ether extract with sodium sulfate, its absorption spectrum was taken and found to be identical with that of leaf xanthophyll. However, the amount present was so small that no crystalline material could be isolated from a concentrated residue, and the portion present represented only a small fraction of the amount that can be isolated from the meal by using the ordinary procedure. A specimen obtained from The American Chlorophyll Company was recrystallized several times from methanol and melted with decomposition at 184–186° (corrected) on rapid heating. The centers of the absorption bands at low temperatures were at 4900, 4580, 4310, and 4050 Å. and lay in the same positions as those of xanthophyll obtained from stinging nettle.

β-Carotene—The petroleum ether extract after removing xanthophyll, alcohol, and water was cooled in an acetone-carbon dioxide bath and the pale yellow, waxy precipitate filtered off. The filtrate was concentrated to about 200 cc. and treated with 100 cc. of acetone. After removal of the precipitate the filtrate was washed with water to remove the acetone. A few hundred cc. of ligroin (b. p. 80°) were added and the solution extracted three times with hot 90 per cent ethanol. The ligroin solution was dried, concentrated to 50 cc., and cooled. Carotene and some fatty substances crystallized. The lipid was removed from the carotene by triturating with a small amount of petroleum ether. The recrystallized carotene had all the physical properties and the same type of absorption spectrum as β-carotene. Unfortunately we were not in a position to determine its optical activity.

We wish to express our appreciation to Dr. Frank M. Schertz for making available to us his wide experience in the field of pigments; to Dr. C. E. Sando, Dr. M. B. Matlack, and Professor R. Kuhn for specimens of materials; and to Dr. O. R. Wulf for numerous spectroscopic suggestions.

Addendum—Since this work was completed there has appeared recently a brief paper by Miller (16) on the absorption spectra of α- and β-carotene and leaf xanthophyll at liquid nitrogen temperatures. In view of the similarity of the methods used and in spite of the fact that a non-polar solvent was used in the case of the carotenes, it seems somewhat surprising that his results should differ so markedly from those of ours. For example, no new bands for these pigments were recorded in his work; moreover, the absorption maxima of "α-carotene," the method of preparation of which was not given, instead of lying closer to shorter wave-lengths than those of β-carotene lay closer to longer wave-lengths, a shift that one would hardly expect to occur by a change of solvent and which is not in agreement with the results of other investigators (8, 17, 18).

BIBLIOGRAPHY

1. Arnold, L. B., and Kistiakowsky, G. B., *J. Am. Chem. Soc.*, **54**, 1713 (1932).
2. Conant, J. B., and Kamerling, S. E., *J. Am. Chem. Soc.*, **53**, 3522 (1931).
3. Bowden, F. P., Morris, S. D. D., and Snow, C. P., *Nature*, **131**, 582 (1933).
4. Matlack, M. B., and Sando, C. E., *J. Biol. Chem.*, **104**, 407 (1934).
5. Karrer, P., and Walker, O., *Helv. chim. acta*, **16**, 641 (1933).
6. Schertz, F. M., *Plant Physiol.*, **3**, 487 (1928).
7. Conant, J. B., and Crawford, F. H., *Proc. Nat. Acad. Sc.*, **16**, 552 (1930).
8. Smith, J. H. C., and Milner, H. W., *J. Biol. Chem.*, **104**, 437 (1934).
9. Zechmeister, L., and Cholnoky, L. V., *Z. physiol. Chem.*, **189**, 159 (1930).
10. Kuhn, R., Winterstein, A., and Kaufmann, W., *Ber. chem. Ges.*, **63**, 1489 (1930).
11. Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Berlin, 237 (1913).
12. Karrer, P., Helfenstein, A., Wehrli, H., Pieper, B., and Morf, R., *Helv. chim. acta*, **14**, 614 (1931).
13. Kuhn, R., and Brockmann, H., *Z. physiol. Chem.*, **206**, 41 (1932).
14. Mackinney, G., and Milner, H. W., *J. Am. Chem. Soc.*, **55**, 4728 (1933).
15. Yamamoto, R., and Muraoka, T., *Sc. Papers Inst. Physic. and Chem. Research*, Tokyo, **19**, 127 (1932).
16. Miller, E. S., *Plant Physiol.*, **9**, 179 (1934).
17. Kuhn, R., and Lederer, E., *Z. physiol. Chem.*, **200**, 246 (1931).
18. Karrer, P., *Chem. Rev.*, **14**, 23 (1934).

THE INFLUENCE OF THE SODIUM AND POTASSIUM CONTENT OF THE DIET UPON THE SODIUM CONCENTRATION OF HUMAN CENTRIFUGED RED BLOOD CELLS

BY ALLAN M. BUTLER AND EATON M. MACKAY

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 16, 1934)

During the course of a study of the influence of potassium chloride administration on the mineral metabolism of man a number of blood specimens were analyzed. Since they have no particular bearing on the other results of the study but are of interest in themselves concerning the sodium content of human red blood corpuscles, we wish briefly to report the analyses here.

From the metabolic data of the experiments the daily intakes of sodium and potassium of two normal adults alone concern us and these are listed in Table I. At the end of each period venous blood was drawn without stasis into closed vessels and there defibrinated with glass beads. The defibrinated blood was then centrifuged for 1 hour at between 2600 and 3000 R.P.M. and analyses made in duplicate on samples from the separated serum and cells. Total base was determined by the method of Van Slyke, Hiller, and Berthelsen (1), calcium and chloride respectively according to the methods of Van Slyke and Sendroy (2, 3), specific gravity by the pycnometer, and water content by drying to constant weight at 110°. Sodium and potassium were determined by a macroapplication of the method of combined sulfates and potassium as potassium chloroplatinate and sodium by difference. The details of the method have been given in Peters and Van Slyke (4).

In Table II we have summarized our analytical data on the inorganic base of human red blood cells and for comparison have given the data of several other investigators. Our total base values for serum tend to be very slightly higher than the average value given by Peters and Van Slyke (5), but considerably above the values reported by Sunderman (6) and Hald (7). Our average

TABLE I

Daily Intake of Na and K

	Period No.	No. of days	Average Na intake	Average K intake	Na:K ratio
			m.-eq. per day	*m.-eq. per day*	
Experiment 1	1	1–6	201	86	2.33
	2	6–12	204	148	1.38
	3	12–18	201	86	2.33
Experiment 2	1	1–6	38	79	0.48
	2	6–12	33	146	0.23
	3	12–18	38	79	0.48
	4	18–22	104	79	1.32

TABLE II

Reported Values of Na, K, Cl, and Total Base in Milli-Equivalents per Liter of Serum and Cells of Normal Men

Author		Cell volume	Serum				Cells			
			Na	K	Cl	Total base	Na	K	Cl	Total base
		per cent								
Kramer and Tisdall (9)	Maximum	43	152	5.1			+6*	114*		
	Minimum	35	140	4.6			−17	105		
	Average	41	146	5.0	101	159†	±0	110		114†
Hald (7)	Average		135	4.5	103	147	24‡	105‡		131‡
Dill, Talbott, and Edwards (10)	Maximum	48	140	4.3	108	152§	17	99	56	
	Minimum	42	137	2.9	103	147	13	89	51	
	Average	45	139	3.6	106	149	15	93	53	
Present paper	Maximum		145	4.7	109	160	23	91	55	129
	Minimum		141	3.6	100	155	15	83	52	119
	Average		143	4.2	103	157	18	88	54	122

* Calculated from analyses of serum and whole blood.

† Sum of the cations.

‡ Calculated from analyses of serum and whole blood, assuming a cell volume of 45 volumes per cent.

§ Sum of cations, assuming 2 milli-equivalents of Mg per liter of serum.

sodium value for serum is 2 milli-equivalents higher than average values obtained by one of us from a large series of analyses of normal human sera by the uranyl zinc acetate method of Barber and Kolthoff as described by Butler and Tuthill (8). It agrees closely with the average value given by Peters and Van Slyke (5) but is distinctly greater than values reported by Sunderman (6) and Hald (7). The data on sodium and potassium concentrations in human red cells from other investigators are very limited and not in agreement. Kramer and Tisdall's (9) cell concentrations were obtained from determinations on serum and whole blood and hematocrit readings of cell volume. Hald's cell concentrations have been estimated by us from her average serum and whole blood values, assuming a cell volume of 45 volumes per cent. Dill, Talbott, and Edwards' (10) cell concentrations were obtained from determinations on centrifuged red cells, as was done in our own analyses. Either method involves the error of incomplete separation of cells from serum, but it seems that Kramer and Tisdall's wide range of concentrations and large negative value reflects an added error in hematocrit reading.

If the average apparent cell sodium concentration from the analyses of Dill, Talbott, and Edwards and ourselves is taken as 16 milli-equivalents per liter, 11.4 cc. of serum would have to be included per 100 cc. of centrifuged cells, if cells completely separated from serum contained no sodium. In view of Hirota's (11) finding that the plastic resistance of human corpuscles is overcome at above 2600 R.P.M. and that at such a rate the real volume of the corpuscles after 60 minutes centrifuging is not more than 1.5 volumes per cent less than the apparent volume, the inclusion of that much serum seems unlikely. The possible adsorption of sodium on the surface of the red cell cannot, however, be ruled out. We are, therefore, uncertain that such corpuscle sodium concentrations as reported mean sodium within the red cell.

In Tables III and IV the analyses of the serum samples of blood drawn at the end of each period as shown in Table I are detailed. Changes in the concentrations of various constituents of the serum in the experiments are slight, owing to the efficiency with which the normal serum concentrations are protected from change. The errors involved in the determinations are indeed almost of the same order of magnitude as the probable changes. The serum

Day of experiment	6th (Period 1)				12th (Period 2)				18th (Period 3)			
	Serum		Cells		Serum		Cells		Serum		Cells	
Protein, *gm. per cent*	8.02				7.12				7.36			
Sp. gr.	1.0193		1.1040		1.0182		1.0936		1.0177		1.0902	
H_2O, *gm. per l.*	926		722		932		724		927		721	
	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O
Na			21.3	29.5	142.0	152.4	16.1	22.2	143.7	155.0	22.7	31.5
K	3.6	3.9	90.3	125.0	4.1	4.4	90.6	125.0	4.6	5.0	90.2	125.0
Ca	5.8	6.3	0.0	0.0	5.0	5.4	0.0	0.0	5.1	5.5	0.0	0.0
Base			122.0	169.0	159.9	171.6	122.5	169.1	155.0	167.3	119.7	166.2
Cl	109.4	118.0	53.5	74.1	100.4	107.7	53.5	73.9	100.0	107.9	54.6	75.7

TABLE IV

Experiment 2, Subject M; Blood Analyses

Day of experiment	6th (Period 1)				12th (Period 2)				18th (Period 3)				22nd (Period 4)			
	Serum		Cells		Serum		Cells		Serum		Cells		Serum		Cells	
Protein, *gm. per cent*	7.25				7.43				6.83							
Sp. gr.	1.0184		1.0983		1.0193		1.0960		1.0177		1.0957		1.0135		1.0926	
H_2O, *gm. per l.*	928		716		933		711		931		713		926		715	
	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O	Per liter serum	Per liter serum H_2O	Per liter cells	Per liter cell H_2O
Na	144.9	156.1	17.5	24.4	143.7	154.1	15.7	22.1	141.0	151.4	14.6	20.5	142.4	153.8	18.1	25.3
K	4.0	4.3	86.4	120.5	4.4	4.7	83.0	116.7	4.7	5.1	89.1	125.0	4.0	4.3	85.8	120.2
Ca	4.9	5.3	0	0	4.6	4.9	0	0	4.7	5.0	0	0	4.9	5.3	0	0
Base			119.0	166.1	156.2	167.5	121.0	170.1	155.6	167.3	129.5	181.5	157.2	169.8	121.6	170.1

values of Experiment 1 (Table III) show concentrations within the normal limits and differences between the periods too small for significant comment. The cell values show concentrations of potassium, total base, and chloride extraordinarily constant. In contrast to these the cell sodium concentration in Period 2 is markedly less than that of Periods 1 and 3. The apparent sodium concentration in cells per liter of cell water was 29.5 milli-equivalents for Period 1 and 31.5 milli-equivalents for Period 3, while for Period 2 it was 22.2. The serum values of Experiment 2 (Table IV) also fall within normal limits and the differences are probably too small for significant comment. The potassium concentration in cells per liter of cell water was not as constant as in Experiment 1. The average value for Experiment 2 was 120.6 compared to 125 milli-equivalents for Experiment 1. During Periods 1 to 3 of Experiment 2, when the sodium to potassium ratio of the diet was low, owing to a low sodium intake, the apparent cell sodium averaged 22.3 milli-equivalents per liter as compared to an average of 30.5 during the two periods of Experiment 1 when the sodium to potassium ratio was high. With 4 days of increased sodium chloride intake the apparent cell sodium concentration rose from the low value of 20.5 milli-equivalents per liter of cell water at the end of Period 3 to 25.3 milli-equivalents per liter of cell water at the end of Period 4.

The consistency of the lower sodium concentrations within the cells as the sodium to potassium ratio in the diet decreased suggests a causative relation. Gerard (12) found the tissue sodium concentration lower in dogs fed a diet low in sodium and high in potassium than in control dogs, though the tissue potassium concentration remained unchanged. Such a dependence as reported here of sodium concentrations in samples of centrifuged red cells on changing sodium to potassium ratios in the diet, while the serum sodium concentration was not affected by change in the diet ratio, would seem to be consistent with the presence of sodium within the red cell.

Table V summarizes the inorganic base composition of centrifuged red cells and the variation of their sodium concentration with the variation of the ratio of sodium to potassium in the diet.

The magnesium content of the cells has been calculated as the difference between the total base and the sum of the sodium, potassium, and calcium concentrations. Greenberg, Lucia, Mackey, and Tufts (13) report the magnesium content of human red blood corpuscles as 5.5 milli-equivalents per liter of cells. Hald (7) gives the concentration as 2.0 milli-equivalents per liter. In the latter case the discrepancy between her figure and ours cannot be explained by a high total base in our analyses, since Hald's cell totâl base exceeded our figures. With such disagreement in cell magnesium concentration the significance of the inverse relation between cell sodium and magnesium concentrations must await further investigation.

TABLE V

Base Composition of Centrifuged Red Cells As Milli-Equivalents per Liter

	Na	K	Ca	Mg*	Total base
Average, per liter cells	18	88	0	16	122
" " " cell H_2O	25	123	0	22	170
" " " " " on high Na:K ratio	31	125	0	12	168
Average, per liter cell H_2O on low Na:K ratio	22	122	0	30	174

* Calculated by subtracting the sum of Na, K, and Ca from the total base.

BIBLIOGRAPHY

1. Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, **74**, 659 (1927).
2. Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **84**, 217 (1929).
3. Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923–24).
4. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **2** (1932).
5. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **1** (1931).
6. Sunderman, F. W., *J. Clin. Inv.*, **9**, 615 (1931).
7. Hald, P. M., *J. Biol. Chem.*, **103**, 471 (1933).
8. Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).
9. Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, **48**, 223 (1921).
10. Dill, D. B., Talbott, J. H., and Edwards, H. T., *J. Physiol.*, **69**, 267 (1930).
11. Hirota, K., *J. Biophysics*, Japan, **1**, 233 (1925).
12. Gerard, P. J., *Compt. rend. Acad.*, **154**, 1305 (1912).
13. Greenberg, D. M., Lucia, S. P., Mackey, M. A., and Tufts, E. V., *J. Biol. Chem.*, **100**, 139 (1933).

THE PARTIAL SYNTHESIS OF RIBOSE NUCLEOTIDES

I. URIDINE 5-PHOSPHORIC ACID

By P. A. LEVENE and R. STUART TIPSON

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 18, 1934)

Natural uridylic acid has been shown to be the phosphoric ester of uridine, a uracil-ribofuranoside. The rate of hydrolysis of the phosphoryl group was found by Yamagawa[1] to be of the same order of magnitude as the rate for muscle inosinic acid. Consequently, since inosinic acid is 5-phospho inosine, it appeared probable that natural uridylic acid might be 5-phospho uridine. However, Levene and Jorpes[2] showed that, on hydrogenation of cytidylic acid, the product (dihydro-cytidylic acid) behaved exactly like yeast adenylic and guanylic acids, both the base and the phosphoryl group being readily split off by dilute acid at 100°. Since it has recently been demonstrated conclusively by Levene and Harris[3] that in yeast adenylic and guanylic acids the phosphoryl group is situated at position (3) of the ribose chain, it seemed permissible to allocate the same formulation to yeast uridylic and cytidylic acids.

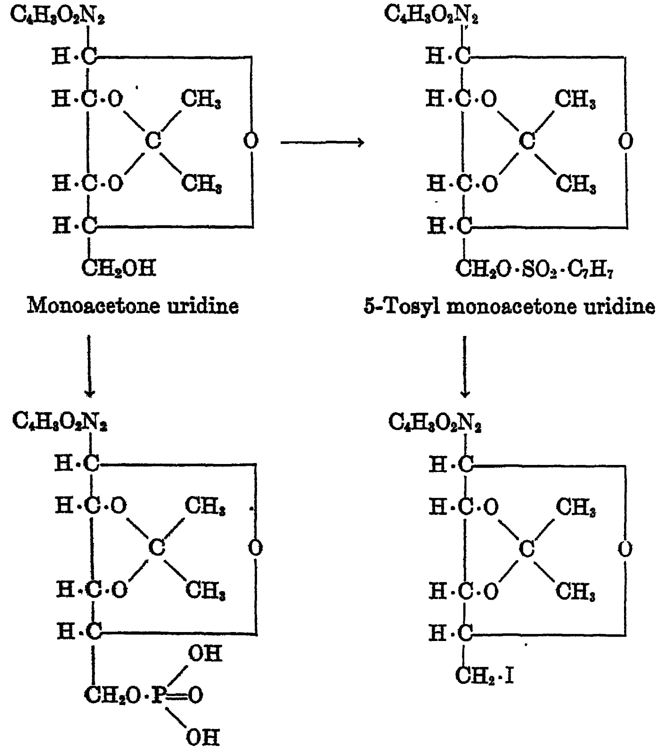
In order to decide whether the natural uridylic acid is or is not 5-phospho uridine, we decided to synthesize 5-phospho uridine. The substance was prepared from monoacetone uridine, a derivative which was shown, in the following way, to have only the hydroxyl group at position (5) free. Monoacetone uridine was treated with a slight excess (1.1 moles) of *p*-toluene sulfonyl chloride in pyridine to give monotosyl monoacetone uridine. Since this compound readily reacts with sodium iodide in acetone to give monoiodo monoacetone uridine, it follows[4,5] that the tosyl group is situated at position (5).

---

[1] Yamagawa, M., *J. Biol. Chem.*, **43**, 339 (1920).

[2] Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **81**, 575 (1929).

[3] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **95**, 755 (1932); **98**, 9 (1932); **101**, 419 (1933).



Monoacetone uridine

5-Tosyl monoacetone uridine

5-Phospho monoacetone uridine

5-Iodo monoacetone uridine

Hence, knowing that uridine is a ribofuranoside[6] and that position (4) is therefore engaged by the oxide ring, it follows that monoacetone uridine is 2,3-monoacetone uracil-ribofuranoside. This agrees, incidentally, with the observations of Levene and Stiller,[7] who have shown that when either ribose or methylribo-

---

[4] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932). Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

[5] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **105**, 419 (1934).

[6] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **101**, 529 (1933).

[7] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933); **104**, 299 (1934).

furanoside condenses with acetone, the acetone residue engages positions (2) and (3) of the ribose chain, leaving position (5) open.

Monoacetone uridine was phosphorylated by treatment with phosphorus oxychloride in pyridine and the resulting 5-phospho 2,3-monoacetone uridine was cautiously hydrolyzed to give 5-phospho uridine. The product had properties quite different from those of natural uridylic acid. In particular, the rate of hydrolysis of the phosphoryl group by 0.1 N hydrochloric acid was much lower, being, indeed, lower than that of any ribose nucleotide yet studied. Even on hydrogenation the rate of hydrolysis, though increased, was lower than that of natural uridylic acid.

There thus remains no doubt that natural uridylic acid is not 5-phospho uridine.

Incidentally, it may be mentioned that the method employed for the synthesis of 5-phospho uridine is being employed in this laboratory for the synthesis of 5-phospho esters of purine nucleosides.

EXPERIMENTAL

Preparation of Monoacetone Uridine—10 gm. of dry, finely powdered uridine were suspended in 250 cc. of acetone (analytical reagent), and then 0.25 cc. of concentrated sulfuric acid and 20 gm. of anhydrous copper sulfate were added. The suspension was shaken at 37° for at least 48 hours. The mixture was now filtered, the copper sulfate well washed with small amounts of pure acetone, and the combined filtrate and washings rendered neutral by shaking with 10 gm. of dry calcium hydroxide powder for 60 minutes.

The mixture was filtered, the calcium salts well washed with acetone, and the combined filtrate and washings evaporated to dryness under diminished pressure. The product invariably crystallized out during this evaporation, giving a colorless, crystalline mass in practically quantitative yield. It is readily recrystallized from absolute methyl alcohol or in the following way. 5 gm. are dissolved in about 60 cc. of boiling acetone, the solution cooled, and pentane added to faint opalescence. On nucleating and standing in the refrigerator, it crystallizes in rosettes of fine needles, m.p. 159–160°.

The crystalline substance was soluble in the cold in the following solvents: absolute ethyl alcohol, absolute methyl alcohol, acetone, pyridine, glacial acetic acid, and water. It was fairly soluble in cold and readily soluble in hot ethyl acetate; sparingly soluble in cold and fairly soluble in hot chloroform; slightly soluble in cold or hot dry ether; and very sparingly soluble in cold or hot carbon tetrachloride, benzene, pentane, or heptane. It was insoluble in methyl iodide but soluble in a mixture of methyl iodide and acetone.

Its analysis was as follows:

5.033 mg. substance: 9.410 mg. CO_2 and 2.565 mg. H_2O
5.915 " " : 0.520 cc. N_2 (753 mm. at 26°)
$C_{12}H_{16}O_6N_2$. Calculated. C 50.68, H 5.7, N 9.86
Found. " 50.98, " 5.7, " 9.94

Its specific rotation was

$$[\alpha]_D^{27} = \frac{-0.32° \times 100}{2 \times 1.012} = -15.8° \text{ (in absolute methyl alcohol)}$$

Preparation of 5-Tosyl Monoacetone Uridine—2 gm. of dry, crystalline monoacetone uridine were dissolved in 12 cc. of dry pyridine, 1.5 gm. (1.1 moles) of tosyl chloride were added, and the mixture shaken until the chloride had dissolved. After standing overnight at room temperature, with the exclusion of atmospheric moisture, 1 cc. of water was added to the brown solution. The resulting solution was kept at room temperature during 30 minutes and the product then isolated, as previously described for the tosylation of dimethyl uridine,[5] giving 3 gm. of a pink, flaky glass which contained no halogen and was soluble in the cold in the following solvents: absolute ethyl alcohol, absolute methyl alcohol, dry ether, acetone, chloroform, benzene, pyridine, ethyl acetate, and glacial acetic acid. It was sparingly soluble in cold but soluble in hot carbon tetrachloride; insoluble in cold but sparingly soluble in hot heptane or water; insoluble in cold or hot pentane.

Its composition was as follows:

4.394 mg. substance: 8.390 mg. CO_2 and 2.100 mg. H_2O
8.160 " " : 0.471 cc. N_2 (754 mm. at 25°)
10.280 " " : 5.410 mg. $BaSO_4$
$C_{19}H_{22}O_8N_2S$. Calculated. C 52.03, H 5.1, N 6.39, S 7.32
Found. " 52.07, " 5.3, " 6.55, " 7.23

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.36° \times 100}{2 \times 1.036} = +17.4° \text{ (in acetone)}$$

Action of Sodium Iodide on 5-Tosyl Monoacetone Uridine—1.0 gm. of dry, glassy tosyl monoacetone uridine was treated with 1 gm. of sodium iodide in 10 cc. of acetone during 2 hours at 100°. The solution remained light brown in color and a large amount of crystalline material was deposited.

The product was isolated in the usual way[5] giving a pale pink, flaky, glass-like substance (yield, 0.9 gm.), which was obtained crystalline by dissolving in a little absolute methyl alcohol and allowing to stand at room temperature for a few hours. It was recrystallized by dissolving in absolute methyl alcohol and adding pentane to faint opalescence, giving colorless crystals having a melting point of 164°. The crystalline substance was soluble in the cold in the following solvents: absolute methyl alcohol, acetone, chloroform, pyridine, ethyl acetate, and glacial acetic acid. It was fairly soluble in cold but quite soluble in hot absolute ethyl alcohol; sparingly soluble in cold but soluble in hot benzene; fairly soluble in cold or hot dry ether; very sparingly soluble in cold or hot pentane; sparingly soluble in cold but fairly soluble in hot carbon tetrachloride or water; insoluble in cold but fairly soluble in hot heptane.

Its composition was as follows:

4.400 mg. substance: 5.915 mg. CO_2 and 1.570 mg. H_2O
8.570 " " : 0.529 cc. N_2 (755 mm. at 22°)
5.914 " " : 3.495 mg. AgI

$C_{12}H_{15}O_5N_2I$.	Calculated.	C 36.54,	H 3.84,	N 7.11,	I 32.2
	Found.	" 36.65,	" 3.10,	" 7.10,	" 32.0

It had the following specific rotation.

$$[\alpha]_D^{28} = \frac{-0.34° \times 100}{2 \times 1.042} = -16.3° \text{ (in acetone)}$$

Methylation of Monoacetone Uridine—4.5 gm. of dry, recrystallized monoacetone uridine were heated under a reflux, with stirring, with 60 cc. of acetone (analytical reagent) until completely dissolved. The solution was cooled and 60 cc. of methyl iodide were added. To the clear solution 2 gm. of dry, finely powdered silver oxide were added. The mixture was stirred and warmed in a bath at 47°. Silver oxide was added in portions of 5 gm. at intervals of 30 minutes until a total of 37 gm. had been added and then the reaction was allowed to proceed during a further 1 hour.[8]

The product was isolated by extraction with chloroform. Evaporation of the chloroform extract gave 4.5 gm. of a colorless, solid, crystalline mass. It may be recrystallized with considerable loss by dissolving in acetone and adding pentane to faint turbidity. It has a melting point of 223–225° and is soluble in cold chloroform, pyridine, and glacial acetic acid. It is fairly soluble in cold acetone, sparingly soluble in cold absolute ethyl alcohol, in methyl alcohol, and in water, but readily soluble in these four solvents on heating. It is insoluble in cold or hot petroleum ether and sparingly soluble in hot dry ether, carbon tetrachloride, benzene, and ethyl acetate.

The crystalline material had the following composition.

5.610 mg. substance: 0.449 cc. N_2 (758 mm. at 22°)
7.455 " " : 6.425 mg. AgI
$C_{13}H_{18}O_6N_2$. Calculated. N 9.40, OCH_3 10.40
Found. " 9.24, " 11.37

Phosphorylation of Monoacetone Uridine—12.5 cc. of dry pyridine were placed in a Pyrex test-tube (65 × 200 mm.) and cooled to −40°. Then 3.5 cc. of phosphorus oxychloride were added and the solution cooled to −40°. To this was added, in six portions with stirring, a cold (at −30°) solution of 10 gm. of dry recrystallized monoacetone uridine in 50 cc. of dry pyridine, the temperature being maintained at −30° to −35°.

After all had been added, the mixture was stirred for a few minutes and then the test-tube was stoppered, transferred to an ice-salt bath, and maintained at −10° to −15° for 2 hours. The mixture set to an almost solid mass. It was now recooled to −35° and 7.5 cc. of 90 per cent aqueous pyridine were added dropwise in such small quantities that, with vigorous cooling and stirring between each addition, the temperature did not rise above −20°.

---

[8] Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, 4, 221 (1931).

The product dissolved very slowly and when heat evolution had ceased, a little ice and 20 cc. of ice water were added. To this solution was slowly added with stirring a solution of barium hydroxide in water, prepared by dissolving 83.5 gm. of barium hydroxide octahydrate in 100 cc. of boiling water and then cooling to 40°. Addition of barium hydroxide was continued until the mixture was faintly alkaline to thymolphthalein, the color of the reaction mixture changing from pale yellow to a vivid orange. A little charcoal was added and the mixture filtered. The charcoal was washed with a little water. On adding the aqueous washings to the main solution a white substance was precipitated.

Without filtering off this precipitate, the mixture was evaporated to dryness under diminished pressure, in order to remove the pyridine. The product was a yellow powder, weighing about 36 gm.

This material was ground with water and the mixture centrifuged. This was repeated many times until the total volume of aqueous extract was 400 cc. and an insoluble material (Fraction A), still containing organic phosphorus, remained. To this aqueous solution were added 400 cc. of acetone with stirring. The white precipitate (Fraction B) was centrifuged off and dried. It weighed 6 gm. This crude material (Fraction B) had a composition corresponding approximately to that of the half barium salt of monoacetone uridine phosphoric acid.

5.196 mg. substance: 18.345 mg. ammonium phosphomolybdate
41.370 " " : 11.900 " $BaSO_4$
$C_{12}H_{16}O_9N_2PBa_{\frac{1}{2}}$. Calculated. P 7.18, Ba 15.91
Found. " 5.13, " 16.92

The aqueous acetone solution was evaporated to dryness, giving a glass (Fraction C) which contained organic phosphate and weighed 20 gm.

The water-insoluble substance (Fraction A) was repeatedly ground with 10 per cent aqueous acetic acid until no more material dissolved. It was then centrifuged and the small amount of insoluble substance washed by stirring with 10 per cent acetic acid and then centrifuging.

Concentrated ammonia was now added to the acetic acid extract until faintly alkaline to neutral red. The mixture was centrifuged and the solution evaporated to dryness. It was freed from ammonium acetate by dissolving in 30 cc. of water and slowly adding 500 cc. of absolute alcohol with stirring. The precipitate was not completely soluble in water. It was boiled with several changes of water, filtered, and the combined filtrate evaporated to dryness. Weight of product (Fraction A_1) = 8.5 gm.

This material (Fraction A_1) was combined with the acetone precipitate (Fraction B) (total weight 14.5 gm.).

Hydrolysis of Fraction C—20 gm. of Fraction C were dissolved in a mixture of 52.5 cc. of water and 22.5 cc. of 5 N sulfuric acid. Without filtering, the mixture was maintained at 75° for 2 hours. It was then cooled and silver carbonate was added until all the chloride had been removed. A little 5 N sulfuric acid was added, if necessary, to render the solution acid to Congo red. The mixture was centrifuged and the precipitate was suspended in 125 cc. of 0.1 N sulfuric acid, well stirred, and again centrifuged.

The combined solution and extract were freed of silver by passing hydrogen sulfide through. The mixture was aerated, filtered, and the filtrate rendered faintly alkaline to phenolphthalein by addition of warm, saturated barium hydroxide solution. The mixture was centrifuged and the precipitate was washed by stirring with water and centrifuging. The combined solution and washings were evaporated to dryness. Yield, 5 gm. The product dissolved readily in 30 cc. of water. To the solution were added 30 cc. of 95 per cent ethyl alcohol and the precipitate was centrifuged off and washed successively with 50 per cent ethyl alcohol, 95 per cent ethyl alcohol, acetone, and dry ether. It was then dried in a vacuum desiccator. Yield, 2 gm.

The white powdery material had the following composition.

9.340 mg. substance: 0.538 cc. N_2 (757.5 mm. at 28°)
5.260 " " : 23.020 mg. ammonium phosphomolybdate
30.925 " " : 13.283 " $BaSO_4$
$C_9H_{11}O_9N_2PBa$. Calculated. N 6.10, P 6.75, Ba 29.89
Found. " 6.49, " 6.36, " 25.28

It had the following specific rotation.

$$[\alpha]_D^{28} = \frac{+0.09^\circ \times 100}{2 \times 1.010} = +4.5^\circ \text{ (in 10 per cent hydrochloric acid)}$$

The combined mother liquor and washings were evaporated to dryness, giving 3 gm. of material.

Hydrolysis of Fraction B + Fraction A_1—14.5 gm. of Fraction B + Fraction A_1 were dissolved in 90 cc. of water and sulfuric acid was added until the solution was faintly acid to Congo red; this required about 10 cc. of 5 N sulfuric acid. A further 25 cc. portion of 5 N sulfuric acid was added, making the solution approximately normal in free sulfuric acid. Without filtering, the mixture was maintained at 75° for 2 hours.

The substance was then isolated as described above, giving 10 gm. of a pale yellow glass, which was dissolved in 25 cc. of water and precipitated by the addition of 25 cc. of 95 per cent ethyl alcohol. The white powdery product was dried as before. It weighed 4 gm. and had the following composition.

8.325 mg. substance: 0.416 cc. N_2 (759 mm. at 24.5°)
21.110 " " : 9.660 mg. $BaSO_4$
$C_9H_{11}O_9N_2PBa$. Calculated. N 6.10, Ba 29.89
Found. " 5.72, " 26.93

It had the following specific rotation.

$$[\alpha]_D^{28} = \frac{+0.07° \times 100}{2 \times 1.017} = +3.44° \text{ (in 10 per cent hydrochloric acid)}$$

The mother liquor and washings were evaporated to dryness giving 4.5 gm. of a colorless glass.

Unlike the barium salt of natural uridylic acid, the substance is very soluble in water.

A portion of the material was dissolved in water and the barium removed quantitatively by addition of 5 N sulfuric acid. Test samples of the solution gave no precipitate with silver nitrate or mercuric chloride. Uranium acetate gave a yellow precipitate. Hopkins' reagent (10 per cent mercuric sulfate in N sulfuric acid) gave a white precipitate and basic lead acetate gave a white curdy precipitate. Neutral lead acetate gave no precipitate, but addition of a few drops of ammonia gave the basic lead precipitate.

A crystalline ammonium salt could not be obtained. The product was a syrup which was insoluble in methyl alcohol and in ethyl alcohol, but soluble in aqueous methyl alcohol.

Preparation of Brucine Salt of Uridine 5-Phosphoric Acid—2 gm. of the barium salt were dissolved in water and the barium was removed quantitatively by addition of 5 N sulfuric acid to about pH 3.5 and then 1 N sulfuric acid dropwise until the barium had been exactly removed. The mixture was centrifuged and the precipitate washed by stirring with water and centrifuging.

To the solution was added a 10 per cent solution of brucine in absolute methyl alcohol until the pH was 7.2 (brom-thymol blue). A trace of insoluble material was filtered off and the solution was evaporated to half the volume and allowed to stand overnight at room temperature, whereupon it crystallized. The crystals were filtered off and dried. Weight, 2 gm. It was recrystallized by dissolving in 15 cc. of warm 33 per cent aqueous methyl alcohol and cooling the solution, giving colorless crystals which softened at 163–165° and melted with foaming at 200°. The crystalline material was soluble in cold pyridine or glacial acetic acid; fairly soluble in cold and readily soluble in hot absolute methyl alcohol or water; sparingly soluble in cold but readily soluble in hot absolute ethyl alcohol; and practically insoluble in either cold or hot dry ether, acetone, chloroform, carbon tetrachloride, benzene, heptane, or ethyl acetate.

It had the following composition.

5.910 mg. substance: 0.420 cc. N_2 (759 mm. at 22°)
2.595 " " : 5.105 mg. ammonium phosphomolybdate
$C_{55}H_{63}O_{17}N_6P$. Calculated. N 7.57, P 2.79
Found. " 8.21, " 2.85

It had the following specific rotation.

$$[\alpha]_D^{24} = \frac{-1.44° \times 100}{2 \times 1.046} = -68.8° \text{ (in dry pyridine)}$$

The specific rotation of the brucine salt of natural uridylic acid was as follows:

$$[\alpha]_D^{24} = \frac{-1.19° \times 100}{2 \times 1.064} = -55.9° \text{ (in dry pyridine)}$$

Hydrogenation of Barium Salt of Uridine 5-Phosphoric Acid—3.5 gm. of the barium salt of uridine 5-phosphoric acid were dissolved in 125 cc. of water. 0.2 gm. of Adams' catalyst was added and the suspension was shaken with hydrogen at a pressure of 40 pounds per sq. inch. The mixture was shaken during 4 days, a little catalyst being added from time to time. A filtered test portion of the solution was, after hydrolysis with dilute mineral acid, strongly reducing towards boiling Fehling's solution. The mixture was filtered and the filtrate evaporated to dryness under diminished pressure. The product was dissolved in a little water and precipitated by the addition of absolute alcohol. It was dried by stirring with absolute alcohol and then with dry ether. The yield was quantitative. The material was a fine, white powder which, unlike the unhydrogenated material, gave a very strong orcinol test.

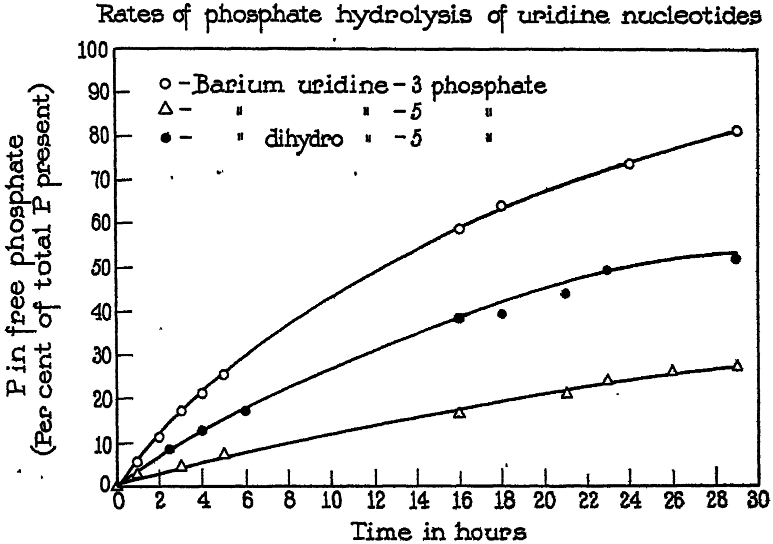


FIG. 1

Rate of Hydrolysis of Phosphoryl Group—The method of preparing the solution of the barium salts of the nucleotides was as follows:

The barium salt was weighed out accurately and dissolved in about 20 cc. of cold distilled water in a 100 cc. volumetric flask. The appropriate volume of N hydrochloric acid was added and the solution was rapidly diluted to 100 cc. with cold distilled water and then cooled in ice.

The respective amounts of barium salt and hydrochloric acid employed were as follows: *barium uridylate* (*natural*) (0.0519 gm. (containing 3.50 mg. of phosphorus), 10.23 cc. of N hydrochloric acid); *barium uridine 5-phosphate* (0.0741 gm. (containing 5.00 mg of phosphorus), 10.32 cc. of N hydrochloric acid); *barium dihydrouridine 5-phosphate* (0.0520 gm. (containing 3.50 mg. of phosphorus), 10.23 cc. of N hydrochloric acid).

Samples of these solutions (approximately 10 cc.) were placed in tubes which were then sealed and heated in the boiling water bath. After the elapse of various intervals of time a tube was removed, rapidly cooled in ice to room temperature, and the amount of free phosphate liberated (calculated as P) in a 5 cc. sample determined by the method of King,[9] a photoelectric colorimeter being used. The results are recorded in Fig. 1.

---

[9] King, E. J., *Biochem. J.*, **26**, 292 (1932).

THE QUANTITATIVE DETERMINATION OF SMALL AMOUNTS OF GONADOTROPIC MATERIAL*

BY PHILIP A. KATZMAN AND EDWARD A. DOISY

(From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis)

(Received for publication, May 21, 1934)

The wide interest shown in the field of sex hormones has led us to devise methods for the detection of minute concentrations of the gonadotropic substances. Several years ago Frank (1) and Loewe (2) reported methods for the determination of the estrogenic substances present in blood and urine, and more recently a number of papers on the excretion of these substances in various conditions has appeared. Gustavson (3) has studied the daily excretion of a normal woman, and Kurzrok (4) has reported results on the urines of a number of women suffering from various types of illness. It would seem that a method of quantitatively determining the substances causing the production of the estrogenic compounds would be of real scientific and perhaps clinical value.

The study of the excretion of gonadotropic substances in conditions unassociated with pregnancy requires the use of a method which is capable of determining exceedingly small amounts of material. A many fold concentration of the active substance must be effected without appreciable loss and the preparations obtained must be tolerated by the test animals. Such a method would be of value in determining the normal excretion in man and animals and in studying the variations which occur during normal and abnormal menstrual cycles as well as in other gynecological conditions.

Although several methods have been developed for the complete extraction of the gonadotropic substance from urine of pregnant

---

* We have been aided by financial assistance from the Theelin Fund administered by the Committee on Grants for Research of St. Louis University.

women (Wiesner and Marshall (5), Zondek, Scheibler, and Krabbe (6), Katzman and Doisy (7), and Davy (8)), no satisfactory procedure has been reported for the quantitative estimation of small quantities of such material which might be present in normal urines. The excretion of gonad-stimulating substances in conditions unassociated with pregnancy has been determined exclusively with untreated urine or by means of Zondek's (9) alcohol precipitation method which gives a 5-fold concentration. A greater concentration, sometimes as high as 20-fold, has been attained by several investigators (Österreicher (10), Hamburger (11), Saethre (12), Schörcher (13), and others), but no investigations have been reported on the accuracy of the determination of the small amounts in normal urine, *i.e.* 5 rat units or less per liter. Even if one assumes that no loss is incurred, a 20-fold concentration would not permit the detection of the active substance in a concentration smaller than 8 to 10 rat units per liter of urine. Further, since the evidence indicates that ethyl alcohol causes appreciable loss of activity (Wiesner and Marshall (5) and Katzman and Doisy (14)) it would appear that this procedure is not suitable for determining the normal excretion of the anterior pituitary-like substance.

Recently we (Katzman and Doisy (15)) described a method for the quantitative determination of small amounts of the gonadotropic substance of urine of pregnancy which had been added to boiled urine. The process was based upon tungstic acid precipitation and removal of the tungstate by means of barium. Wiesner and Marshall (5) and Zondek, Scheibler, and Krabbe (6) have found that phosphotungstic acid quantitatively precipitates the active material of urine of pregnancy but they have not reported studies on the recovery of the small quantities of the active substance which occur in normal urine. Since the tungstic acid method gave such a surprisingly good recovery of minute quantities (15), it did not seem worth while to investigate the phosphotungstic acid precipitation.

Though the tungstic acid-barium process gave satisfactory recoveries of added gonadotropic substance, two undesirable features remained: (1) an occasional extract which was decidedly toxic to the test animals; and (2) the necessity of a large centrifuge for the separation of the tungstic acid precipitate.

The first difficulty was partially overcome by the substitution of solid brucine for barium salts in the decomposition of the tungstic acid precipitate and the second was eliminated by the use of the benzoic acid precipitation followed by filtration instead of centrifugation. Furthermore, the extracts prepared by the benzoic acid process were less toxic than those prepared by the tungstic acid procedure.

To complete the study it was necessary to determine whether the processes devised for extracts of urine of pregnancy could be applied to the recovery of the active substance of gland extracts and the gonadotropic material of urine of castrates. There appears to be rather satisfactory evidence that the extracts from these three sources exert somewhat different effects. Since our interest lay chiefly in the determination of the quantitative excretion of non-pregnant individuals, it was important to determine whether the proposed methods would permit the quantitative recovery of the gonadotropic substances of the pituitary.

The recognition of activity adopted by us differs somewhat from the criteria used by some investigators. Our preparations and the extracts of urine were assayed by their capacity to produce opening of the vagina and cornification of the vaginal epithelium. Injections were made at 9.00 a.m. and 4.00 p.m. on 3 successive days. Rats 21 days of age were used; if a cornified smear did not appear before the 29th day less than 1 rat unit had been injected.

Various reports on the thermolability of the gonadotropic substances have been confirmed in our laboratory. Nevertheless, we have prepared extracts of boiled urine which gave negative results at assay and have also made extracts of normal urine which were found to lose their potency on boiling. It has, therefore, seemed safe to test our processes by studying the recovery of measured quantities of gonadotropic substances added to urines which had previously been boiled for at least 1 hour.

Lest our report leave an erroneous impression in the reader's mind, we believe that the success of our processes probably depends upon the adsorption of the active material on some protein-like substance that is precipitated by tungstic or benzoic acid. With aqueous solutions of highly purified extracts of urine of pregnancy the procedures used give a very poor recovery, but the same preparations added to urines are quantitatively recovered.

EXPERIMENTAL

Tungstic Acid Process—In this procedure (Katzman and Doisy (15)) a 24 hour urine sample to which had been added 10 cc. of 10 per cent sodium tungstate and 10 cc. of 0.5 per cent casein was made acid to Congo red paper with dilute H_2SO_4. The precipitate was collected by centrifugation, washed with acetone, freed from acetone by reduced pressure, and dissolved by adding dilute NaOH until the mixture was faintly alkaline to phenolphthalein. Solutions of $BaCl_2$ and $Ba(OH)_2$ were then added in the proportion necessary to prevent the solution from becoming acid, until precipitation was complete. The precipitate was removed by centrifugation, and the excess barium precipitated by Na_2SO_4, the $BaSO_4$ removed, and the solution neutralized with dilute acetic acid. In this manner it was possible to concentrate the original sample to approximately 10 cc., representing a 100-fold concentration, without an appreciable loss of activity.

The reliability of the method was determined by the recovery of known, small amounts of the gonadotropic substance of urine of pregnancy which had been added to boiled urine. Of eight experiments in which from 4 to 5 rat units were added to boiled urines, the recovery was from 75 to 110 per cent in seven tests. The average of these values was 92 per cent.

Extending our work with the tungstic acid method to urine of pregnancy (7) it was found that the barium precipitation, which was also used for the decomposition of the phosphotungstate precipitate by Wiesner and Marshall (5), and Zondek, Scheibler, and Krabbe (6) was less satisfactory than the treatment with certain inexpensive alkaloids, notably brucine. Furthermore, we have found that the casein may be omitted; the precipitate of the protein substances of urine being adequate to precipitate completely the gonadotropic material of normal urine.

Tungstic Acid-Brucine Process—With this information at hand we attempted to improve the tungstic acid-barium procedure by the following modifications. The urine to which has been added 20 cc. of 10 per cent Na_2WO_4 per liter is acidified with H_2SO_4 until it is acid to Congo red paper. The precipitate is collected by centrifugation, washed with acetone, freed from acetone by suction, and suspended in a small volume of H_2O. Solid brucine is added with thorough stirring until the reaction is neutral or slightly alkaline to litmus. After standing for several hours in the cold room, to allow the brucine to decompose the precipitate and to precipitate the tungstate, the mixture is centrifuged. The extraction with brucine is repeated twice more with short extraction periods. The combined extracts are added to 10 volumes of cold acetone and a little sodium acetate solution is added to facilitate precipitation (Evans (16)). This treatment serves to concentrate the active material in the precipitate and to retain the brucine in solution. The precipitate is collected by centrifugation, freed from acetone, and dissolved in the desired volume of H_2O. By this procedure it is possible to attain a concentration greater than 100-fold and the extracts appear to be less toxic than those obtained by the tungstic acid-barium method.

Our experiments show that one extraction with aqueous brucine does not result in uniformly good recovery of the extract of pregnancy urine which had been added to boiled urine. This is undoubtedly due to the bulkiness of the precipitate which retains an appreciable amount of the solution. With three extractions the recovery is more uniform and fairly good, though perhaps not so satisfactory as in the benzoic acid process. In the two recovery experiments with fairly large amounts (16.6 rat units) of gonadotropic material from beef hypophyses, recoveries of 130 and 110 per cent were obtained. This may have been due to variations in the test animals or possibly to a synergetic action between the gland material and the urinary substance, as suggested by Evans, Simpson, and Austin (17) and Leonard and Smith (18). The second possibility seems improbable in view of the fact that the urine had been boiled for over 1 hour prior to the addition of the extract.

Benzoic Acid Process[1]—From the standpoint of ease of manipulation and completeness of recovery of small quantities of gonadotropic material we have found that a modification of our benzoic acid adsorption method (14) is the most satisfactory procedure.

---

[1] After we had obtained a considerable amount of information by the tungstic acid-barium process, Dr. E. T. Engle informed us of his success in the use of the benzoic acid adsorption for removing small quantities of gonadotropic material from urine. Consequently we have studied the benzoic acid process to determine suitable conditions to secure quantitative results. We take this opportunity to express our appreciation to Dr. Engle.

In most of our experiments, urine which had been chilled in the refrigerator overnight was used. The cold urine is acidified to pH 5 with acetic acid and for each liter, 75 cc. of acetone saturated with benzoic acid are added with vigorous stirring. The precipitated benzoic acid is collected on a Buchner funnel, washed with a saturated aqueous solution of benzoic acid, and dissolved in cold acetone. The acetone-insoluble material, which may be collected either by centrifugation or by suction filtration on a small funnel, is washed with acetone and dissolved in dilute NaOH. The pH is not permitted to exceed 9. Any insoluble material is removed by centrifugation and the extract is neutralized to pH 7 to 8 with dilute acetic acid. A second or even a third extraction of the precipitate with dilute sodium hydroxide may be advisable. The extract may be concentrated by adding 10 volumes of cold acetone, collecting the precipitate, freeing it from acetone, and dissolving it in the desired volume of H_2O. Since this procedure makes possible a 200-fold concentration, an entire 24 hour urine sample can be injected into a single immature rat, permitting the detection of 1 rat unit in that sample. We have noted no toxic effects when one-half of the extract of a 24 hour urine is injected but some preparations are toxic in amounts twice as large as this.

Table I shows the effectiveness with which gonadotropic material of urine of pregnancy is recovered from chilled boiled urine by this method. For the preparations in the first section of Table I two precipitations of benzoic acid were used. The precipitated benzoic acid was not washed, nor were the extracts concentrated by acetone precipitation. Amounts ranging from 3 to 22 rat units were, in most instances, completely recovered.

From the data presented in the second section of Table I it may be seen that one precipitation with benzoic acid completely removed the added active material. Further, washing the benzoic acid precipitate or concentrating the extract by acetone precipitation or a combination of both caused little loss. Amounts ranging from 1.3 to 330 rat units were satisfactorily recovered by this procedure. The method is thus applicable to a wide range of concentrations but its reliability for the quantitative recovery of the large amounts of gonadotropic substance present in pregnancy urines was not investigated.

We have also studied the effect of using urine and acetone at room temperature, because the chilling of these materials may be an inconvenience if adequate refrigeration facilities are not available. The data in the third section of Table I show that fairly

TABLE I

Benzoic Acid Adsorption. Recovery of Gonadotropic Substance of Urine of Pregnancy Added to Boiled Urine

Volume of boiled urine	No. of experiments	Quantity of material added	Variations in quantity recovered	Average quantity recovered		Remarks
With 2 precipitations of benzoic acid						
cc.		*rat units*	*rat units*	*rat units*	*per cent*	
500	2	3.2	>2.9	>2.9	>90	Benzoic acid precipitates not washed, extracts not concentrated by acetone precipitation
500	5	4.4	2.2- 4.7	3.8	86	
500	5	6.6	>4.2- 8.3	6.4	97	
500	1	11.0	14.7		130	
500	1	15.4	13.3		80	
500	1	22.0	22.0		100	
With 1 precipitation of benzoic acid*						
500-750	5	1.3	<1 ->1.1	1.1	85	
500-750	4	3.2	>2.6->3.3	>2.9	>90	
500-750	4	4.8	>4.2->4.8	>4.6	>96	
1000	1	80.0	>66		>82	
1000	1	165.0	110 -165		70-100	
1000	1	333.0	220 -330		66-100	
With unchilled urine and acetone (1 precipitation)						
750	8	3.3	2.0- 3.0	2.4	76	Urine not chilled
500	7	3.3	2.0- 4.2	3.1	94	Acetone " "

* The following data were also obtained on the sixteen preparations, in the second section of the table, as follows:

2, not washed or concentrated. Average recovery 100 per cent
3, washed and concentrated. Average recovery 90 per cent
6, not washed but concentrated. Average recovery 84 per cent
5, " concentrated but washed. " " 86 " "

good and uniform recoveries result even under these conditions. However, we believe that the chilling is preferable whenever it is feasible to carry it out.

Since the gonadotropic substance of the hypophysis is believed to differ from that excreted during pregnancy and since it is presumed that the material excreted in conditions unassociated with pregnancy is of hypophyseal origin, it was deemed advisable to attempt to recover the active substance of gland extracts after it had been added to boiled urine. As can be seen from Table II this material is recovered by the benzoic acid method just as effectively as is the substance from urine of pregnancy. Unfortunately in those cases where a recovery >70 per cent is recorded the extracts were used up before the assays were fully completed. We feel that the recovery in these instances was actually better than is indicated in Table II. In the two experiments performed (Table II) we have also been able to recover quantitatively from boiled urine added known amounts of the gonadotropic substance obtained from the urine of castrated women.

TABLE II

Benzoic Acid Process. Recovery of Anterior Pituitary Gland Gonadotropic Material Added to Boiled Urine

Volume of boiled urine	Quantity of material added	Quantity of material recovered		No. of precipitations with benzoic acid	Benzoic acid precipitate washed
cc.	*rat units*	*rat units*	*per cent*		
500	25	18.7	90	2	–
500	16.6	22.0	130	2	–
500	3.3	>2.2	>70	1	–
500	3.3	>3.3	>100	1	–
500	6.6	>4.2	>70	1	–
500	6.6	>4.4	>70	1	–
750	3.3	2.6	80	1	+
750	3.3	3.3	100	1	+
600	2.8	>2.4	>86	1	–
600	5.6	3.5	60	1	–
600	3.4*	3.0	88	1	–
600	8.5*	>8.5	>100	1	–

* Gonadotropic substance from the urine of ovariectomized women.

The determination of the gonadotropic substances of urine is beset with some difficulties that may not be apparent to the casual reader. Zondek and Aschheim (19) and Zondek (20) have reported the existence of separate follicle-stimulating and luteinizing substances in urine and Fevold, Hisaw, and Leonard (21) and Wallen-Lawrence (22) have separated extracts of the anterior lobe into two fractions, one of which produces follicular growth and the other luteinization. The urine of ovariectomized women and women past the menopause, according to the studies of Zondek (23), Leonard (24), Riley, Brickner, and Kurzrok (25), and Hamburger (26) contains chiefly the follicle-stimulating factor. Recently, Leonard and Smith (18) have shown that extracts obtained from urines after menopause produce a response in hypophysectomized females which differs from the reaction produced by extracts of urine of pregnancy. It seems then that at least two gonadotropic substances exist.

Upon the basis of the production of vaginal canalization and estrus our experiments show rather definitely that the benzoic acid procedure will determine quantitatively the estrus-stimulating substance of urine of pregnancy, castrated women, or during the menopause. With both the luteinizing and follicle-stimulating substances present in an unknown proportion, as in extracts of the anterior lobe, the recovery is also approximately quantitative. This may be fortuitous or it may be due to the procedure used for assay. Our data show that we can recover quantitatively the potency of extracts which are primarily luteinizing or follicle-stimulating when these extracts are assayed by their capacity to induce estrus in immature rats. Since we do not use changes in the weights of the ovaries as the criterion of activity but instead the production of estrus, our data for normal urines possibly represent the lump sum of all gonadotropic substances present. As methods improve it will be interesting to determine the proportions of follicle-stimulating and luteinizing material in normal urine.

Fevold, Hisaw, Hellbaum, and Hertz (27) have reported that benzoic acid precipitates most of the luteinizing factor and little of the follicle-stimulating factor from sheep pituitary extracts. In our recovery experiments we have not observed such fractionation. The response to the gonadotropic material from each source was apparently unaltered by being subjected to the recovery process. The gland extracts after being recovered still produced the typical large ovaries, loaded with corpora lutea and follicles, while the ovaries of the animals treated with the recovered material from urine of pregnancy were small in spite of the presence of lutein bodies. Further, the follicle-stimulating substance of the urine of ovariectomized women was completely recovered by this procedure.

TABLE III

Gonadotropic Material in Normal Urine

	Tungstic acid method			Benzoic acid method		
	No. of subjects	No. of urine samples	Excretion per 24 hrs.	No. of subjects	No. of urine samples	Excretion per 24 hrs.
Adult males	8	6	<4 ->19 m.u.	9	10	<1->2 r.u.
		7	<3 - 7 r.u.			
Girls before puberty	3	3	<2.4- <3.7 " *			
Boys before puberty	6	8	<2.7- <4.2 m.u.*	4	4	<1 r.u.
		6	<2.3- <3.2 r.u.*			
Boys after puberty	1	1	7 r.u.			
Girls after puberty	1	1	4 "			
Menopause	9	6	9 - 26 m.u.			
		3	5.4- >5.4 r.u.			
				36 liters urine; 3-4 r.u. per liter		
Adult females	AD	76	<3.2- 14 m.u.	AD	72	<1- 6 r.u.
	CD	51	<3.3- 10 "	LK	33	<1- 2.3 "
	GD	23	<3 ->10 "	W	13	<3->7 m.u.
	AJ	25	<3.2->16 "			
	A	13	<3 ->20 "			
	B	19	<2.2->31 "			
	W	40	<2.9->14 "			

M.u. = mouse units; r.u. = rat units.

* No positive reactions were obtained with these urines.

Excretion of Gonadotropic Substance in 24 Hour Urines—Preliminary data on the normal daily excretion of gonadotropic material are presented in Table III. Preparations obtained by the tungstic acid method were for the most part assayed on immature mice, while the benzoic acid extracts were tested on immature rats. These data agree with our earlier findings (14) that the mouse is more sensitive with respect to the production of cornified cells in the vaginal smears than the rat to the extent that 1 rat unit is equal to 3 to 4 mouse units.

Although we have not been able to demonstrate the presence of this factor in all specimens of urine from normal men, amounts as large as 19 mouse units have been found in some samples. Schörcher (13) and Ehrdhard and Ruhl (28) were unable to find prolan in the urine of children and young people between the ages of 7 days and 20 years while Neumann and Peter (29) reported that it occurred quite constantly in infants from 1 to 11 days of age but only occasionally in older children. Saethre (12) found in some cases that girls excreted as much as 55 mouse units per liter. Our own data indicate that between the ages of 4 years and puberty little or none of the gonadotropic substance is found in the urine, but that at puberty an increased excretion occurs.

The presence of this material in the urine of menstruating women is also disputed. Zondek (30) working with mixed samples found that a maximum excretion is reached just before and a fall occurs immediately after menstruation. Kaufmann and Mühlbock[2] have also reported that the urine of women with normal menses contains a gonadotropic substance. However, Österreicher (10) found this to be true only in a few cases, while Kurzrok (4) could not find it at all. Frank, Goldberger, and Spielman (31) and Frank (32) have shown that the greatest concentration in the blood occurs in the early part of the cycle. Our own results usually show a maximum excretion occurring about the time of ovulation and sometimes also during menstruation. Between these maxima the values fall to 3 mouse units or 1 rat unit or less.

In agreement with Zondek (33), Jeffcoate (34), Winter (35), Österreicher (36), Hamburger (11), and Saethre (12) we have found an increased excretion after the menopause. However, our values are lower than those reported by some of the other investigators. In the urine of ovariectomized women we have found larger quantities of a gonadotropic substance than are found in the urine of normally menstruating women. The excretion here seems to be quite variable, in some cases reaching a level of 300 mouse units or 75 rat units per 24 hour period.

---

[2] Kaufman, C., and Mühlbock, O., *Klin. Woch.*, 12, 1480 (1933).

DISCUSSION

Although considerable work has been done on the determination of the excretion of gonadotropic substances in conditions unassociated with pregnancy, it is surprising that no one has investigated the reliability of the methods employed for this purpose. It is obvious that such a determination is necessary before one can rely on the data obtained on the normal excretion and the variations which occur in pathological disturbances in which the excretion of such material is affected.

We believe that the recovery of measured amounts of gonadotropic material added to boiled urine constitutes a satisfactory measure of such reliability. Extracts by the benzoic acid method have been prepared from boiled urine to which no gonadotropic material had been added to ascertain whether additional ovary-stimulating activity was imparted to the extracts obtained in our recovery experiments. Contrary to the results reported by Gostimirovic (37), such extracts had very little effect either on the ovaries or uterus. There was, perhaps, a slight increase in the weight of the ovaries but in no instance was opening of the vagina or estrus produced. Further, extracts of the urine of normal and ovariectomized women and of women in the menopause lost their activity on boiling, indicating that estrogenic material was absent.

It would appear, therefore, that we have at hand a method which is adequate for measuring small as well as fairly large quantities of gonadotropic material. Since this method is equally efficient in recovering from boiled urine the added estrus-stimulating substance of anterior lobe extracts or of the urines of pregnant or ovariectomized women, it should be applicable to the study of the normal excretion of ovary-stimulating substances. Our investigation of this phase of the problem is rather inadequate because of the limited number of subjects studied. However, the results are fairly consistent and indicate that rather definite limits can be established for the variations of the normal excretion. We have not attempted the study of the excretion of gonadotropic material in various gynecological conditions and in other disturbances related to abnormal hypophyseal activity, but it would seem that the possibilities are quite alluring.

Leonard and Smith (18) have recently reported that extracts of the urine of women after the menopause produce ovarian changes in the hypophysectomized rat quite different from those resulting from extracts of the urine of pregnant women. Our own observations on immature rats not hypophysectomized indicate that the ovarian changes produced by extracts of gland, urine of pregnancy, and after the menopause differ. While that amount of material from urines of women after the menopause or of ovariectomized women which is just sufficient to induce opening of the vagina and estrus (*i.e.* 1 rat unit) produces an appreciable follicle stimulation and enlargement of the ovaries, a corresponding quantity of an extract of fresh anterior lobes elicits these phenomena to a much greater degree. 1 rat unit of the former produces an average ovarian enlargement of about 5 mg., whereas the same amount of the latter induces an increase of 20 to 30 mg. Further, increasing the dosage of the urinary products to as much as 10 rat units yields only an additional increase in ovarian weight of 5 to 10 mg. It would thus appear that the quality responsible for the growth of the ovaries which is abundantly present in the gland extracts is strikingly absent in the preparations made from urines of women past the menopause and ovariectomized women. In this respect these extracts resemble the gonadotropic material of urine of pregnancy. On the other hand, enormous follicles are often produced but corpora lutea and blood spots are usually absent. It is possible that we are dealing largely with the follicle-stimulating principle and that only occasionally the luteinizing factor is present.

In many instances, the extracts obtained from the urine of normal adult women had little effect upon the weight of the ovaries of the test animals. When injected in quantities sufficient to induce opening of the vagina and estrus, the weight of the ovaries was not appreciably greater than that of the control rats. Yet smaller amounts of the same extracts produced an appreciable enlargement, even though estrus did not occur. Furthermore, the injection of an extract of an entire 24 hour urine sample occasionally failed to induce either vaginal opening or estrus yet was effective in enlarging the ovaries. Since we are certain that estrogenic material (with respect to the castrate rodent) was not present in these extracts, these unexpected findings cannot be attributed to this factor. These confusing results indicate that a more extensive study should be made of the gonadotropic substance of normal urine.

SUMMARY

1. Two methods for the quantitative estimation of small amounts of gonadotropic substance from either urine of pregnancy or gland extracts are described. One is based on tungstic acid precipitation and removal of the tungstate by means of barium or brucine, the other on benzoic acid adsorption. The efficiency of the methods was tested by recovery experiments in which known small amounts of gonadotropic material were added to boiled urine.

2. Preliminary data are presented on the daily excretion of gonadotropic material by adult men, and women, menstruating, and after the menopause, and prepuberal girls and boys.

3. The extracts of urine of women during menopause and castrates appear to produce a response in the immature rat somewhat different from the response to extracts of urine of pregnancy or of anterior lobes.

We wish to express our appreciation to Miss Corinne Dewes for her efficient assistance with the assays.

BIBLIOGRAPHY

1. Frank, R. T., Frank, M. L., Gustavson, R. G., and Weyerts, W. W., *J. Am. Med. Assn.*, **85**, 510 (1925).
2. Loewe, S., *Klin. Woch.*, **4**, 1407 (1925).
3. Gustavson, R. G., and Green, D. F., *J. Biol. Chem.*, **105**, xxxiv (1934).
4. Kurzrok, R., *Endocrinology*, **16**, 361 (1932).
5. Wiesner, B. P., and Marshall, P. G., *Quart. J. Exp. Physiol.*, **21**, 147 (1931).
6. Zondek, B., Scheibler, H., and Krabbe, W., *Biochem. Z.*, **258**, 102 (1933).
7. Katzman, P. A., and Doisy, E. A., *Proc. Soc. Exp. Biol. and Med.*, **31**, 188 (1933).
8. Davy, L., *Endocrinology*, **18**, 1 (1934).
9. Zondek, B., *Klin. Woch.*, **9**, 1209 (1930).
10. Österreicher, W., *Klin. Woch.*, **12**, 538 (1933).
11. Hamburger, C., *Klin. Woch.*, **12**, 934 (1933).
12. Saethre, H., *Klin. Woch.*, **12**, 1727 (1933).
13. Schörcher, F., *Klin. Woch.*, **10**, 2221 (1931).
14. Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.*, **98**, 739 (1932).
15. Katzman, P. A., and Doisy, E. A., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1188 (1933).

16. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, **58**, 545 (1933).
17. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, **58**, 561 (1933).
18. Leonard, S. L., and Smith, P. E., *Am. J. Physiol.*, **108**, 22 (1934).
19. Zondek, B., and Aschheim, S., *Klin. Woch.*, **7**, 1453 (1928).
20. Zondek, B., *Klin. Woch.*, **9**, 2285 (1930); **9**, 245 (1930).
21. Fevold, H. L., Hisaw, F. L., and Leonard, S. L., *Am. J. Physiol.*, **97**, 291 (1931).
22. Wallen-Lawrence, Z., *J. Biol. Chem.*, **105**, xcvii (1934).
23. Zondek, B., *Klin. Woch.*, **9**, 393 (1930).
24. Leonard, S. L., *Am. J. Physiol.*, **98**, 406 (1931).
25. Riley, A. R., Brickner, R. M., and Kurzrok, R., *Bull. Neurol. Inst. New York*, **3**, 53 (1933).
26. Hamburger, C., *Ugesk. Læger*, 287 (1933); abstracted in *Ber. ü. Ges. Gynäk. u. Geburtsh.*, **25**, 219 (1933).
27. Fevold, H. L., Hisaw, F. L., Hellbaum, A., and Hertz, R., *Am. J. Physiol.*, **104**, 710 (1933).
28. Ehrdhard, K., and Ruhl, H., *Arch. Gynäk.*, **154**, 294 (1933).
29. Neumann, H. O., and Peter, F., *Z. Kinderheilk.*, **52**, 24 (1931).
30. Zondek, B., *Klin. Woch.*, **10**, 2121 (1931).
31. Frank, R. T., Goldberger, M. A., and Spielman, F., *Proc. Soc. Exp. Biol. and Med.*, **28**, 999 (1931).
32. Frank, R. T., *J. Am. Med. Assn.*, **97**, 1852 (1931).
33. Zondek, B., *Klin. Woch.*, **11**, 1839 (1932).
34. Jeffcoate, T. N. A., *Lancet*, **1**, 662 (1931).
35. Winter, E. W., *Arch. Gynäk.*, **151**, 201 (1932).
36. Österreicher, W., *Klin. Woch.*, **11**, 813 (1932).
37. Gostimirovic, D., *Zentr. Gynäk.*, **57**, 2476 (1933).

THE SYNTHESIS OF CYSTINYLDIGLYCINE AND CYSTINYLDIALANINE

By JULIUS WHITE*

(*From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor*)

(Received for publication, May 10, 1934)

The synthesis of a simple peptide in which the carboxyl group of cystine is bound in the peptide linkage has not yet been reported. Hopkins (1) has obtained the diketopiperazine of cystine and glycine by boiling glutathione with water. Kendall, Mason, and McKenzie (2) have obtained glutamic acid and a dipeptide of cystine and glycine by the hydrolysis of glutathione in water at 62°. The peptide was not isolated but was identified as the trinitrotoluene salt. Bergmann and Zervas (3) have prepared the ethyl ester of dicarbobenzoxycystinyldiglycine.

Compounds of this type possess properties which would be of considerable interest. They would offer an excellent opportunity for a study of their fate in the animal organism and for comparison with the fate of related cystine derivatives already known. Likewise an enzymatic study would be of interest. It should also be possible to apply this method to the synthesis of glutathione, and such work is at present being carried out.

In the present paper a modification of the method of Bergmann and Zervas (3) has been successfully applied to the synthesis of two cystinyl peptides, cystinyldiglycine and cystinyldialanine. A study of the biological behavior of these two peptides will be reported in a later paper.

EXPERIMENTAL

Dicarbobenzoxycystinyl Dichloride—10 gm. of the finely pulverized dicarbobenzoxycystine prepared according to the method of Bergmann and Zervas (3) were suspended in 60 cc. of anhydrous ether in a 125 cc. Erlenmeyer flask and cooled in an ice-salt bath. To this were added at once 8.2 gm. of finely powered phosphorus pentachloride. This was then shaken vigorously. After several minutes of shaking solution was complete. The cooling and shaking were continued for 15 minutes, during which time the acid chloride separated out as beautiful needles. 10 to 15 cc. of dry ether were added and the whole was filtered. The precipitate was then washed with a small quantity of ether. The yield was quantitative. Since the acid chloride decomposed in the presence of moisture and air, it was prepared only as it was needed. The last traces of ether were not removed until the acid chloride was actually used. This was done by placing small quantities on a porous plate and allowing the ether to evaporate spontaneously.

---

* National Research Council Fellow in Medicine.

Dicarbobenzoxycystinyldiglycine—3.5 gm. of glycine were dissolved in 30 cc. of N sodium hydroxide and cooled in an ice bath. To this was gradually added with shaking, the acid chloride obtained from 10 gm. of dicarbobenzoxycystine. If, after each addition of the acid chloride, the acid chloride failed to dissolve, more N sodium hydroxide was added (about 10 cc. portions). After all the acid chloride was added, the solution was allowed to stand in the ice bath for 1 hour. It was then acidified with 5 N hydrochloric acid from which the product precipitated out. This was filtered off and recrystallized from dilute alcohol. It had a melting point of 161–163°. The yield was 10 gm. (87.5 per cent of the theoretical amount).

Analysis—0.2257 gm. substance: 14.3 cc. 0.1 N HCl (Kjeldahl)
0.2220 " " : 0.1640 gm. $BaSO_4$
$C_{26}H_{30}O_{10}N_4S_2$. Calculated. N 9.00, S 10.29
Found. " 8.87, " 10.14

Cystinyldiglycine—2 gm. of dicarbobenzoxycystinyldiglycine were suspended in 20 cc. of concentrated hydrochloric acid in a 100 cc. Erlenmeyer flask. This was heated on a water bath. The temperature of the water bath was not allowed to go above 70°. As the temperature gradually rose, the material went into solution with the evolution of carbon dioxide and the formation of an oil on the surface. This oil imparted a turbid appearance to the mixture but in about 30 minutes the solid material was completely dissolved. The mixture was diluted to 150 cc. with water and the oil was removed by extraction with ether. The aqueous layer was then slowly added with shaking to a solution of 5 gm. of phosphotungstic acid dissolved in a minimum amount of water. This mixture was cooled in an ice bath for 1 hour and the precipitate filtered off. The precipitate was suspended in 100 cc. of water, 1 cc. of concentrated hydrochloric acid was added, and the whole was extracted with two 50 cc. portions of a 1:1 ether-amyl alcohol mixture. The aqueous layer was separated and treated with an excess of magnesium oxide and thoroughly shaken. This mixture was filtered and the filtrate was concentrated under reduced pressure until crystals began to form. The residue was dissolved in a small quantity of water (5 to 10 cc.) and filtered into 400 cc. of ethyl alcohol. The precipitate which formed was filtered off and rubbed with alcohol until there was no further evidence of chlorides. The yield was 0.6 gm. (53 per cent of the theoretical amount). The hydrochloric acid could also be removed by treatment with silver oxide and sulfuric acid and subsequent removal of the silver ion with hydrogen sulfide followed by the removal of the sulfate ion with barium hydroxide. This latter method was more troublesome. Attempts to remove the carbobenzoxy group by catalytic reduction with hydrogen and platinum or palladium black as a catalyst were unsuccessful.

Analysis—0.1450 gm. substance: 16.3 cc. 0.1 N HCl (Kjeldahl)
0.2018 " " : 0.2675 gm. $BaSO_4$
$C_{10}H_{18}O_6N_4S_2$. Calculated. N 15.81, S 18.07
Found. " 15.80, " 18.16

Estimation of NH_2-N (*4*)
Calculated. 0.2070 gm. required 11.69 cc. 0.1 N HCl
Found. 0.2070 " " 11.7 " 0.1 " "

Estimation of COOH and Other Acidic Groups
Calculated. 0.2070 gm. required 11.69 cc. 0.1 N NaOH
Found. 0.2070 " " 11.75 " 0.1 " "

Dicarbobenzoxycystinyldialanine—The same method used for the preparation of dicarbobenzoxycystinyldiglycine was employed here. The reaction of dicarbobenzoxycystinyl dichloride [illegible] alanine was carried out under the same conditions. The product was difficult to crystallize, so it was used for the following reaction in the crude form.

Cystinyldialanine—The hydrolysis was carried out in concentrated hydrochloric acid as described for cystinyldiglycine. The temperature in this case, however, was allowed to rise to 85°. The yield was 0.65 gm. (53 per cent of the theoretical amount).

Analysis—0.1004 gm. substance: 10.5 cc. 0.1 N HCl (Kjeldahl)
0.1146 " " : 0.1375 $BaSO_4$
$C_{12}H_{22}O_6N_4S_2$. Calculated. N 14.66, S 16.7
Found. " 14.59, " 16.5

Estimation of NH_2-N
Calculated. 0.1085 gm. required 5.67 cc. 0.1 N HCl
Found. 0.1085 " " 5.7 " 0.1 " "

Estimation of COOH and Other Acidic Groups
Calculated. 0.1085 gm. required 5.67 cc. 0.1 N NaOH
Found. 0.1085 " " 5.73 " 0.1 " "

Sullivan (5) has stated that his colorimetric reaction for cystine is specific with the exception of methylcystine. Both cystinyldiglycine and cystinyldialanine responded to this color reaction. The equivalent of 1 mg. of cystine as the peptide gave a far more intense color than did 1 mg. of cystine. This color was not the characteristic one obtained with cystine but was magenta in appearance. Hence it would seem impossible to determine cystine in the presence of either of these derivatives.

BIBLIOGRAPHY

1. Hopkins, F. G., *J. Biol. Chem.*, **84**, 269 (1929).
2. Kendall, E. C., Mason, H. L., and McKenzie, B. F., *J. Biol. Chem.*, **88**, 409 (1930).
3. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
4. Harris, L. J., *Proc. Roy. Soc. London, Series B*, **95**, 500 (1923).
5. Sullivan, M. X., and Hess, W. C., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1599 (1929).

On page 144, Vol. 106, No. 1, August, 1934, last paragraph, first sen[illegible] read *Sullivan's (5) colorimetric reaction for cystine has been shown to be specific with the exception of cystine dimethyl ester (Brand, E., Harris, M. M., and Biloon, S., J. Biol. Chem.,* **86**, *315 (1930))* for *Sullivan (5) has stated that his colorimetric reaction for cystine is specific with the exception of methylcystine.*

HUMAN MILK STUDIES

XV. THE NON-PROTEIN NITROGEN CONSTITUENTS

By BETTY NIMS ERICKSON, MARGARET GULICK, HELEN A. HUNSCHER, and ICIE G. MACY

(*From the Research Laboratory of the Children's Fund of Michigan and the Children's Hospital of Michigan, Detroit*)

(Received for publication, May 7, 1934)

The chemical composition and nutritional value of human milk are generally appraised in terms of the percentage of fat, lactose, proteins, and ash constituents. The non-protein nitrogen compounds, however, which are included in the figures for protein and often disregarded, may have important significance both in the physiology of milk secretion (1) and in the nourishment of the infant (2) even though they are present in little more than traces. Recent work has indicated that some of these relatively minute components may reflect more specifically than the gross composition certain dietary changes (3) and pathological conditions of the mammary gland itself (4).

There appear to be two possible sources of the non-protein nitrogen constituents in the milk. They may be derived from the synthetic or even catabolic metabolism of nitrogenous compounds in the milk or in the gland itself. This source is suggested by certain observations of abnormalities in the non-protein nitrogen fraction of milk contaminated with bacilli (4–6) and of milk produced during the existence of pathological conditions in the mammary gland (7–9). On the other hand, the non-protein nitrogen constituents may filter from the blood directly into the milk as indicated by observations which show that they normally occur in approximately the same concentration in milk as in blood (10–17). Even though the individual non-protein nitrogen constituents may originate directly from the blood, it is possible that they may either maintain equilibrium with the corresponding composition of the blood or may vary in concentration depending upon the rapidity and the extent of their utilization in the synthesis of milk proteins and other nitrogenous compounds.

More information upon the concentration of this heretofore neglected fraction, the non-protein nitrogen, in milk secreted under normal and abnormal conditions, may give enlightenment upon the source of the constituents of this fraction and even upon the physiology of milk secretion. For instance, if the non-protein nitrogen constituents were normally of metabolic origin in the gland, one might expect considerable fluctuation in their concentration at different times, subject to local conditions of the gland, the stage of protein synthesis at the time the milk is removed, and the activity or work of the gland as approximated by the milk volume.

Accordingly, the magnitude of variation of certain non-protein nitrogen constituents in milk has been observed in the first and last halves of the nursing period, at different times of the day and night, during several successive days, and at intervals throughout lactation. In addition, the non-protein nitrogen partition, including the total non-protein nitrogen, urea, amino acids, uric acid, creatine, and creatinine of milk during the initial days of lactation and in an instance of high fever of a subject in early lactation has been studied in considerable detail.

Procedure

Information concerning the organization and plan of the studies has been reported previously: a discussion of the subjects, of the method of completely removing the milk from the breasts, and of dividing the milk from both breasts at a nursing period into samples representative of the complete first and last halves of nursing (18); a statement of the method of removing the milk almost simultaneously from the two breasts (19); an outline of the methods of collecting and analyzing the milk at every 4 hour interval throughout the day and night (20); and an account of the plan of the successive day study periods including daily analyses based on composite 24 hour samples of milk removed from both glands at every 4 hour interval during the day and night (21).

Methods of Analysis

The methods developed by Denis (10, 11) for analysis of the non-protein nitrogen constituents of milk were used for all of the studies except those on the milk during the initial days of Lactation C and during a fever of Subject 7. For these a recently developed modified procedure of the tungstic acid removal of proteins (22) and the Van Slyke gasometric methods for total non-protein nitrogen (23), urea (24), and amino acids (25) were used.

Non-Protein Nitrogen Constituents of Milk during Initial Days of Lactation—Pronounced abnormalities and fluctuations in the composition of milk during the initial days of lactation have been observed (26–29). The anomalous composition has been attributed both to intrinsic causes of teleological significance to the new-born and to physiological influences; *i.e.*, a flushing out of accumulated milk constituents in the adjustment of the congested gland to the processes of a regulated milk flow (30). That the

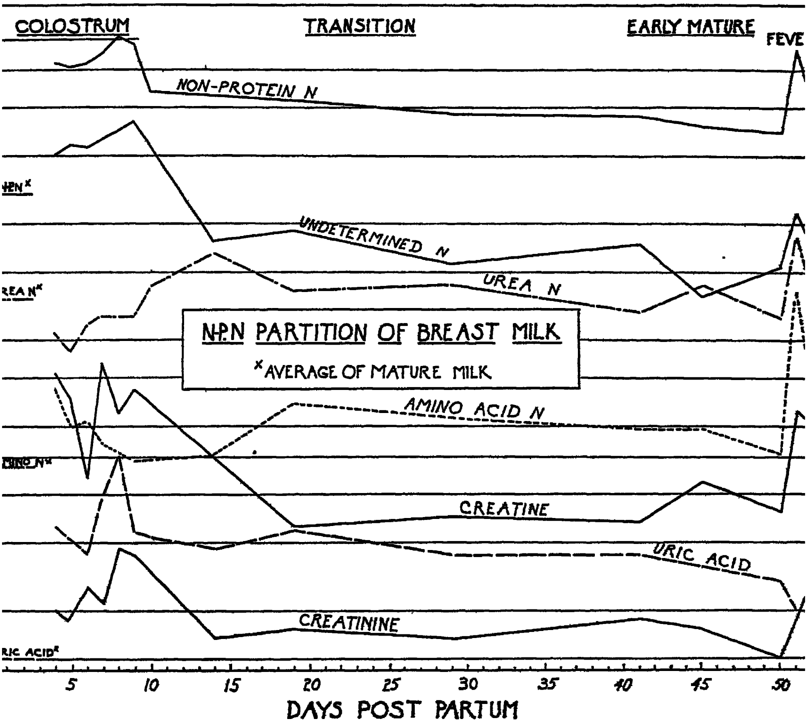


CHART I. Non-protein nitrogen partition of human milk in the initial days of Lactation C and during a fever of Subject 7.

mammary gland does synthesize and store milk components long before lactation begins has been shown by the presence of casein in the gland as early as the 24th week of pregnancy (31). There has been variance of opinion regarding the length of time before the gland does secrete a milk of normal composition (27, 32). However, periods in early lactation have been classified as follows (27): colostral stage (1 to 12 days), during which the milk remains appreciably abnormal; transitional stage (13 to 30 days), during which the milk gradually approaches that of normal composition; mature (1 to 9 months), throughout which an average normal composition is maintained. The length of time required for the establishment of a regulated milk flow appears to differ among lactating women.[1]

Since the non-protein nitrogen has been found to be affected by pathological disturbances of the mammary gland itself (7–9), a study of this fraction in early lactation, when the gland is changing from a quiescent to an actively secretory organ, is of physiological significance. The non-protein nitrogen partition of colostrum is strikingly irregular and abnormal with an elevated total non-protein nitrogen, uric acid, creatine, and creatinine as shown by data (in Chart I) on Subject 7 for the first 11 days of Lactation C and at frequent intervals thereafter until the 8th week post partum. The averages (in mg. per 100 cc.) of the non-protein nitrogen constituents in the milk for the first 12 days of lactation and in the preceding mature Lactation B (3 to 7 months) on the same subject are respectively: total non-protein nitrogen 52.9 and 38.8; urea nitrogen 11.2 and 15.1; uric acid 4.1 and 1.9; amino acid nitrogen 6.6 and 6.2; creatine 7.1 and 1.0; creatinine 2.2 and 1.1. The total non-protein nitrogen, uric acid, creatine, and creatinine were still somewhat augmented during the transitional period as shown by average values (mg. per 100 cc.) of 42.7, 3.0, 4.2, and 1.8 respectively. In fact, some of the constituents even at the 7th week of lactation had not assumed the normal concentration of the preceding mature lactation. The unusually high total non-protein nitrogen of colostrum is in accord with experiments which have been made on animals (10), while on the contrary, augmented urea and amino acids, which have been reported to be characteristic of colostral milk (10, 33), were not observed.

---

[1] A comprehensive report of the factors influencing human milk production as based upon extensive observations and the secretion of human milk is given in Macy, I. G., Hunscher, H. A., Donelson, E., and Nims, B., *Am. J. Dis. Child.*, **39**, 1186 (1930).

Milk which has been allowed to remain in the udder for a few days before removal (34, 35), and milk from infected mammary glands have been noted to resume the characteristics of colostral milk, particularly in the non-protein nitrogen partition (7–9). Such a resemblance suggests that the abnormal non-protein nitrogen characteristics of colostrum likewise may be due to local conditions of the gland itself. On the other hand observations of similar alterations in the non-protein nitrogen partition of milk secreted during body fever suggest that certain factors, other than the gland, may contribute to the unusual non-protein nitrogen of colostrum.

Within 24 hours after an elevated temperature of this woman, her milk changed exceedingly with an augmented non-protein nitrogen, creatine, and creatinine, in which respect it resembled colostrum; other abnormalities were exhibited such as a high amino acid and urea nitrogen, with values increased nearly 100 per cent above those of the preceding day (Chart I). The values (mg. per 100 cc.) of the constituents in the milk on the 1st day of fever in comparison with those of the preceding day were increased as follows: total non-protein nitrogen 34.4 to 56.9; urea nitrogen 11.3 to 18.7; amino acid nitrogen 5.1 to 13.6; uric acid 2.0 to 2.4; creatine 3.6 to 6.1; and creatinine 1.5 to 1.9. Of the 21.4 mg. of undetermined non-protein nitrogen, 12.4 mg. were found to be in the form of bound amino acid nitrogen.

Such anomalies in the non-protein nitrogen constituents of milk secreted during fever suggest that they may be related to concomitant changes in the blood which are associated with general bodily disturbances. Febrile conditions have been noted to be accompanied by a high blood non-protein nitrogen and by other abnormal changes which are a clinical index of accelerated bodily protein catabolism (36). The similarities in the non-protein nitrogen fraction of colostrum and of milk secreted during fever (see Chart I) stimulate a more thorough investigation of the singular composition of colostrum in relation to unusual conditions of the blood accompanying parturition. The blood at parturition and during the puerperium has been shown to be abnormal with a high total non-protein nitrogen (37, 38), urea (37–39), uric acid (37, 40, 41), and creatine (42, 43); these are irregularities indicative of bodily protein catabolism, which are presumably associated with the involution of the uterus. It appears then that in addition to alterations imposed by the transition of the mammary gland to an actively secretory organ, the anomalous composition of colostral milk may be, in part, a reflection of an abnormal blood picture which in turn is associated with the nitrogen metabolism of the puerperium. These indications, supported by data (Table I) which generally show a close correspondence between the composition of the blood and milk in early and late lactation, furnish evidence that the non-protein nitrogen constituents in the milk may normally originate directly from the blood.

TABLE I

Non-Protein Nitrogen Constituents in Blood and Milk† (Mg. per 100 Cc.)*

Subject No.	Lactation period	Stage of lactation	Non-protein N		Urea N		Amino acid N		Uric acid	
			Blood	Milk	Blood	Milk	Blood	Milk	Blood	Milk
6	A	Colostrum	32.7	53.3	13.3	11.1	7.4	4.2		
		Transition	33.8	47.2	11.3		6.3	4.6		
		Early mature	37.0	41.0	11.4			6.4		
7	A	Colostrum	40.0	42.5	11.6		12.7	4.7		
		Transition	51.3	37.5	26.7		6.4	5.2		
		Early mature	36.4	28.0	18.9			4.3		
	C	Colostrum	52.9	51.7	24.5	11.1		6.1	4.9	2.8
6	B	6th mo.	35.0	30.3	17.3	16.7	5.6	6.3	3.2	1.8
		9th "	30.9	25.6	15.0	16.2	5.7	6.6	2.9	2.0
		14th "	28.4	30.5	13.2	17.8	6.2	6.1	3.2	2.9
7	B	6th "	32.9	30.6	15.8	15.5	7.8	5.4	3.5	2.1
		8th "	30.3	27.3	17.3	16.0	8.0	7.2	3.2	1.5
8	B	6th "	53.3	59.2	25.5	17.4	8.4	7.7	4.9	2.0
		10th "	53.8	30.2	26.2	20.4	5.5	5.8	3.9	2.3
		14th "	63.8		21.2	15.9	12.7	4.8	4.1	2.8

* Fasting sample.

† Composite 24 hour samples.

Milk Representative of First and Last Halves of Nursing Period— The numerous studies upon cows, concerning the actual time of milk synthesis in relation to the removal of milk from the udder, have been reviewed previously (18). In general they indicated that milk formation is most rapid immediately after milking and proceeds at a diminishing rate as the milk accumulates in the gland. If there is an accelerated synthesis of protein after a portion of the accumulated milk has been removed from the breasts, as has been indicated by previous observations (18), the influence on the concentration of the free and bound amino acids in the milk is of physiological significance.

These studies included observations on the milk of three women at different stages in lactation. From sixteen comparisons of the total non-protein and amino acid nitrogen in the milk representative of the first and last halves of nursing, we have found differences in total non-protein nitrogen from 0 to 9 mg. per 100 cc., values being higher in the last half in some cases and lower in the last half in others. However, an average of these figures gives a mean of 0.6 mg. or 2 per cent less total non-protein nitrogen and 0.2 mg. or 4 per cent less amino acid nitrogen per 100 cc. of milk in the last half of the nursing period. The slightly diminished non-protein nitrogen of 0.6 mg. per 100 cc. does not account for the increased protein corresponding to a rise in total nitrogen of 5 mg. per 100 cc. in the last half of the nursing period (18). The results indicate that the amino acids may be replaced almost immediately by others from the blood, or that there may be a progressively rapid release of previously synthesized protein with the progression of nursing.

Comparison of Milk from Right and Left Breasts—Composite 24 hour collections of milk from the right and left breasts were analyzed separately. The total non-protein nitrogen (mg. per 100 cc.) differed only slightly in the milk from the two breasts: left, 34.8, right 33.7; and left 31.2, right 29.9 of two women respectively; an average difference of 1.2 mg. or 3 per cent. The amino acid nitrogen (mg. per 100 cc.) likewise differed very little in the milk from the two glands: left 3.6, right 3.7; and left 4.7, right 5.0; an average difference of 0.2 mg. or 4 per cent. Although the two breasts differed in volume output of milk, very little difference was found in the two nitrogen fractions. The data, although few, indicate that the non-protein nitrogen in the milk may be determined normally by the amount in the blood rather than by local conditions and activity of the mammary gland.

The results agree with similar studies reported from this laboratory (19), which have shown that under carefully controlled conditions of milk removal and analysis, the two mammary glands of a woman secreted milk uniform in the percentage composition of fat, protein, lactose, total ash, calcium, and phosphorus. Such similarity of percentage composition was observed even though the two glands differed in volume of milk produced, thus differing in gland capacity and activity. These results, with the additional data indicating similarity in the percentage of non-protein and amino acid nitrogen in the milk from the two breasts, give evidence against theories of derivation of milk constituents from degeneration of cells in the mammary gland (19).

TABLE II

*Diurnal Variation of Total Non-Protein Nitrogen and Amino Acid Nitrogen**
(Mg. per 100 Cc.)

		Non-protein N					Amino acid N				
Subject No.	Study No.	Maximum	Time	Minimum	Time	Per cent of difference	Maximum	Time	Minimum	Time	Per cent of difference
6	V	36.2	6 p.m.	31.2	10 p.m.	16	5.6	6 p.m.	2.8	6 a.m.	100
	VI	36.9	10 "	30.6	10 a.m.	21	5.7	2 "	3.8	10 "	50
7	III	31.4	10 "	27.7	10 "	13	4.6	6 "	3.2	6 "	44
	IV	34.4	6 "	22.8	6 "	51	4.3	All hrs. except 10 p.m.	4.2	10 p.m.	2
8	VII	35.8	6 "	28.3	6 "	27	4.8	10 p.m.	4.1	2 and 6 a.m.	17
9	I	34.5	2 a.m.	31.1	10 p.m.	11	7.0	6 "	4.7	2 a.m.	49
Average...........................						23					44

* Analyses made at 6 a.m., 10 a.m., 2 p.m., 6 p.m., 10 p.m., and 2 a.m.

Diurnal Variation in Total Non-Protein Nitrogen and Amino Acid Nitrogen—The studies included analyses of total non-protein nitrogen and amino acid nitrogen at every 4 hour interval throughout the day and night during 6 days. The maximal and minimal values, the time at which they occurred, and the per cent of difference between the maximum and minimum for each day studied are given in Table II. Maximal and minimal values of non-protein nitrogen during the day differed by 3.4 to 11.6 mg. per 100 cc. with an average difference of 6.3 mg. or 23 per cent. Maximal and minimal values of amino acid nitrogen differed by as much as 2.8 mg. per 100 cc. with an average difference of 1.5 mg. or 44 per cent. The non-protein nitrogen and

TABLE III

Day to Day and Month to Month Variations in Non-Protein Nitrogen Constituents (Mg. per 100 Cc.)*

Subject No.	Study No.	No. of days	Non-protein N			Urea N			Amino acid N			Uric acid		
			Maximum	Minimum	Per cent of difference	Maximum	Minimum	Per cent of difference	Maximum	Minimum	Per cent of difference	Maximum	Minimum	Per cent of difference
Day to day variations														
6	I	10	37.2	29.3	27				5.6	4.2	33			
7	I	10	37.0	24.7	50				5.7	3.6	58			
6	VIII	5	31.2	27.9	12	18.9	16.4	15	6.4	5.7	12	1.9	1.5	27
6	IX	5	27.9	25.6	9	18.1	16.2	12	6.7	5.4	24	2.3	1.8	28
6	X	5	35.0	30.5	15	20.9	17.8	17	11.3	6.1	85	3.2	2.9	10
7	VIII	5	31.6	29.2	8	16.5	13.5	22	7.9	5.3	49	2.3	1.8	28
	IX	5	30.9	26.4	17	19.2	15.9	21	7.2	5.4	33	1.8	1.4	29
8	IV	5	41.1	33.7	22	21.7	17.4	25	7.7	4.0	93	2.3	1.9	21
	V	5	33.7	29.0	16	22.3	19.2	16	7.0	5.6	25	2.4	2.0	20
Average..................					19			18			46			23
Month to month variations														
6	10 mos.		41.2	17.3	138				4.9	3.6	36			
7	10 "		42.4	23.6	80				6.4	3.6	78			
6	7 "		60.4	25.6	136	23.5	16.2	45	7.0	4.3	63	3.3	1.5	120
7	7 "		41.0	23.8	72	17.2	12.7	35	10.6	4.7	126	2.1	1.5	40
8	7 "		59.5	27.5	116	22.3	16.2	38	7.5	5.5	36	2.4	1.3	85
Average..................					108			39			68			82

* Composite 24 hour samples.

amino acid nitrogen tended to be highest in the afternoon or evening and to be least concentrated in the morning milk. It is interesting to note that the trend of diurnal variation of these constituents is similar to that observed for total nitrogen (20).

The observations are in accord with others from this laboratory, which have demonstrated that, independent of such factors as food, rest, activity, family surroundings, and stage of lactation, there are characteristic changes during the day in milk volume and in percentage composition of fat, total solids, total nitrogen, total ash, calcium, and phosphorus (20). The regularity of fluctuation of these milk constituents intimates some peculiarity of mammary gland physiology which causes a rhythmic diurnal variation of activity and synthesis of individual milk constituents. Recent evidence indicates that diurnal variations may occur not only in the percentage composition of individual blood constituents (44–47) but also in the chemical structure of blood proteins (48, 49).

Variations in Non-Protein Nitrogen Constituents from Day to Day, from Month to Month, during Lactation, and Among Individual Women—Studies included analyses of composite 24 hour samples of milk for 5 to 10 consecutive days at different times in the lactation cycle, as well as composite 24 hour samples at monthly intervals on three women during two successive lactation periods (Table III).

The amino acid nitrogen was most variable from day to day with an average of 46 per cent difference between high and low values; the urea and total non-protein nitrogen were somewhat less variable with an average per cent of difference of 18 and 19 respectively. The diurnal fluctuations in total non-protein nitrogen and amino acid nitrogen either exceeded or were of the same magnitude as those from day to day (Table IV). The fluctuations of total non-protein nitrogen, urea, amino acids, and uric acid from month to month greatly exceeded those variations among consecutive days, and at different times of the day. Both the monthly studies and consecutive day periods at different times in lactation indicated certain trends in the change of the non-protein fraction as lactation progressed; *i.e.*, a diminution of total non-protein nitrogen and an increase of urea nitrogen.

The components of the non-protein nitrogen fraction varied more widely among the milks of different women[2] than they did in the milk of one woman throughout an entire lactation period (Table IV). Similar results have been observed in other constituents of milk, which, although varying from day to day and during lactation, fluctuate about a level characteristic to the individual woman's milk (21). It is likely that individuality in the non-protein nitrogen fraction of milk may be related to similar individual characteristics in the blood composition (3).

TABLE IV

Magnitude of Variation of Non-Protein Nitrogen Constituents

Study	No. of determinations	Non-protein N	Urea N	Amino acid N	Uric acid
Average difference between maximum and minimum (*mg. per 100 cc.*)					
During single day	36	6.3		1.5	
From day to day	55	5.5	3.0	2.2	0.4
" mo. to mo. during lactation	41	25.3	5.9	3.0	1.2
In milk of different women	11	16.0	10.0	8.3	4.5
Average difference between					
Right and left breasts	4	1.2		0.2	
First " last halves of nursing period	32	0.6		0.2	
Average per cent of difference between maximum and minimum					
During single day	36	23		44	
From day to day	55	19	18	46	23
" mo. to mo. during lactation	41	108	39	68	82
In milk of different women	11	73	105	208	161
Average per cent of difference between					
Right and left breasts	4	3		4	
First " last halves of nursing period	32	2		4	

DISCUSSION

The data reported herein indicate that under normal physiological conditions the non-protein nitrogen constituents seem to assume a concentration in the milk similar to that in which they occur in the blood. Even colostrum with its characteristically abnormal composition of non-protein nitrogen components seems to reflect corresponding abnormalities in the blood. Likewise, the abnormal milk secreted during fever appears to reproduce disturbances of the nitrogen partition of the blood.

---

[2] The authors appreciate the helpful cooperation of Dr. Raymond Hoobler and Mrs. Pinkett for collecting and supplying 24 hour samples of milk from eleven women.

The theory of the origin of the non-protein nitrogen components directly from the blood is compatible with recent observations on physicochemical aspects of milk secretion (50). In view of the isotonicity of blood and milk it has been proposed that at no time during synthesis does the osmotic pressure of the fluid in the mammary gland rise above that of the blood. Hence, as osmotically active amino acids are used in synthesis of osmotically inactive colloid milk protein, they are constantly replaced in order to maintain osmotic equilibrium with the blood. It is not known to what extent other non-protein nitrogen constituents of blood are used for synthesis of more complex nitrogenous substances in the milk. The large undetermined non-protein nitrogen fraction of milk offers a fertile field for investigation of the complex nitrogenous products such as simple peptides or bound amino acid compounds which are not of peptide nature.

The evidence of the direct origin of non-protein nitrogen compounds in the milk from the blood under normal conditions does not vitiate indications that certain abnormal conditions involving infection of the mammary gland may bring about drastic alterations in the non-protein nitrogen partition of the milk. In fact, this evidence supports the suggestion that the non-protein nitrogen may serve as an index of abnormalities (22). If the milk exhibits anomalous non-protein nitrogen characteristics, particularly in established mature lactation, one may look for similar abnormalities in the blood, which are associated with bodily disturbances, for infections in the gland itself (7–9), or for bacterial contamination of the milk (4–6). The average composition (mg. per 100 cc.) of the non-protein nitrogen partition of milk for three women in established lactation (3rd to 7th months) was found to be as follows: total non-protein nitrogen 37.7, urea nitrogen 17.9, amino acid nitrogen 6.3, and uric acid 2.1.

SUMMARY

Observations have been made on the total non-protein nitrogen and amino acid nitrogen of human milk in the first and last halves of the nursing period, and at 4 hour intervals throughout the day and night.

The non-protein nitrogen partition of the milk, including total non-protein nitrogen, urea, amino acids, uric acid, creatine, and creatinine, has been studied in human milk from day to day during several successive days, during the initial days of early lactation, at monthly intervals throughout mature lactation, and in an instance of a high fever of a subject in early lactation.

The non-protein nitrogen partition of the milk in the initial days of lactation is strikingly irregular and abnormal with an elevated non-protein nitrogen, uric acid, creatine, and creatinine.

The milk secreted during a fever exhibited similar striking abnormalities with an augmented total non-protein nitrogen creatine, creatinine, urea, and amino acid nitrogen.

The total non-protein nitrogen and amino acid nitrogen were practically of the same concentration in the milk from the first and last halves of the nursing period, and in the milk from the right and left breasts.

The total non-protein nitrogen and amino acid nitrogen varied appreciably throughout the day and night. A rhythmical diurnal fluctuation appeared to exist with maximal values in the afternoon or evening, and minimal values in the morning. The concentration of the non-protein nitrogen constituents in the milk varied from day to day, more so throughout lactation, and most widely among different women.

The data indicate that under normal physiological conditions the concentration of non-protein nitrogen constituents in the milk is similar to that observed in the blood. Under abnormal conditions of bodily disturbances, drastic alterations may occur in the non-protein nitrogen partition of the milk, alterations which may serve clinically as an index of such bodily disturbances as reflected in the blood.

BIBLIOGRAPHY

1. Macy, I. G., Outhouse, J., Long, M. L., Brown, M., Hunscher, H., and Hoobler, B. R., *J. Biol. Chem.*, **74**, xxxi (1927).
2. Courtney, A. M., and Brown, A., *Arch. Dis. Child.*, **5**, 36 (1930).
3. Perkins, A. E., *Ohio Agric. Exp. Stat., Bull. 446*, 115 (1930); *Chem. Abst.*, **24**, 5057 (1930).
4. Smejkal, O., *Milchwirtschaft. Forsch.*, **14**, 251 (1932); *Nutrition Abst. and Rev.*, **2**, 700 (1933).
5. Supplee, G. C., *J. Dairy Sc.*, **1**, 313 (1917–18).
6. Frazier, W. C., and Rupp, P., *J. Bact.*, **16**, 65 (1928); **21**, 263 (1931).
7. Davies, W. L., *J. Dairy Research*, **4**, 142 (1932).
8. Kieferle, F., *Milchwirtschaft. Forsch.*, **14**, 567 (1933); *Nutrition Abst. and Rev.*, **3**, 44 (1933).
9. Neseni, R., *Milchwirtschaft. Forsch.*, **14**, 549 (1933); *Nutrition Abst. and Rev.*, **3**, 45 (1933).
10. Denis, W., and Minot, A. S., *J. Biol. Chem.*, **38**, 453 (1919).
11. Denis, W., Talbot, F. B., and Minot, A. S., *J. Biol. Chem.*, **39**, 47 (1919).
12. Taylor, W., *Biochem. J.*, **16**, 611 (1922).
13. Cary, C. A., *J. Biol. Chem.*, **43**, 477 (1920).
14. Venuti, A., *Riv. clin. pediat.*, **27**, 308 (1929); *Abst. Am. J. Dis. Child.*, **42**, 646 (1931).
15. Sato, M., and Murata, K., *J. Agric. Chem. Soc. Japan*, **9**, 1 (1933); *Chem. Abst.*, **27**, 2222 (1933).
16. Blackwood, J. H., *Biochem. J.*, **26**, 772 (1932).
17. Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, **18**, 53 (1914).
18. Macy, I. G., Nims, B., Brown, M., and Hunscher, H. A., *Am. J. Dis. Child.*, **42**, 569 (1931).
19. Brown, M., Macy, I. G., Nims, B., and Hunscher, H. A., *Am. J. Dis. Child.*, **43**, 40 (1932).
20. Nims, B., Macy, I. G., Brown, M., and Hunscher, H. A., *Am. J. Dis. Child.*, **43**, 828 (1932).
21. Nims, B., Macy, I. G., Hunscher, H. A., and Brown, M., *Am. J. Dis. Child.*, **43**, 1062 (1932).
22. Erickson, B. N., Stoner, N., and Macy, I. G., *J. Biol. Chem.*, **103**, 235 (1933).
23. Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926–27).
24. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 449 (1929).
25. Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).
26. Lowenfeld, M. F., Widdows, S. T., Bond, M., and Taylor, E. I., *Biochem. J.*, **21**, 1 (1927).
27. Schloss, E., *Monatschr. Kinderh.*, **9**, 636 (1911); **10**, 499 (1912).
28. Schlossmann, A., *Arch. Kinderh.*, **30**, 288 (1900).
29. Hammett, F. S., *J. Biol. Chem.*, **29**, 381 (1917).
30. Provan, A. L., *Welsh J. Agric.*, **4**, 141 (1928); *Chem. Abst.*, **23**, 1670 (1929).
31. Cutler, O. I., and Lewis, J. H., *Am. J. Physiol.*, **103**, 643 (1933).

32. Gardner, J. A., and Fox, F. W., *Practitioner*, **114**, 153 (1925).
33. Giaume, C., *Riv. clin. pediat.*, **26**, 356 (1928).
34. Petersen, W. E., and Rigor, T. V., *Proc. Soc. Exp. Biol. and Med.*, **30**, 257 (1933).
35. Hohlfeld, M., *Jahrb. Kinderh.*, **138**, 185 (1933).
36. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **1**, 294–298 (1932).
37. Harding, V. J., Allin, K. D., and Van Wyck, H. B., *J. Obst. and Gynec. Brit. Emp.*, **31**, 595 (1924).
38. Rowe, A. W., Gallivan, D., and Matthews, H., *Am. J. Physiol.*, **95**, 592 (1930).
39. Parsons, H. T., *J. Biol. Chem.*, **88**, 311 (1930).
40. Bunker, C. W. O., and Mundell, J. J., *J. Am. Med. Assn.*, **83**, 836 (1924).
41. Slemons, J. M., and Bogert, L. J., *J. Biol. Chem.*, **32**, 63 (1917).
42. Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, **34**, 5 (1918).
43. Plass, E. D., *J. Biol. Chem.*, **56**, 17 (1923).
44. Nicolaysen, R., *Biochem. Z.*, **248**, 275 (1932).
45. Dupré, E. F., and Semeonoff, E., *J. Biol. Chem.*, **94**, 341 (1931–32).
46. Porcher, C., and Maynard, L., *Lait*, **10**, 601, 765 (1930); *Chem. Abst.*, **25**, 328 (1931).
47. Allcroft, W. M., *Biochem. J.*, **27**, 1820 (1933).
48. Aszódi, Z., *Biochem. Z.*, **252**, 387 (1932).
49. Schenck, E. G., and Kunstmann, H. K., *Z. physiol. Chem.*, **215**, 87 (1933).
50. Blackwood, J. H., and Stirling, J. D., *Biochem. J.*, **26**, 1127 (1932).

A REINVESTIGATION OF THE PHENOMENON OF A FIRST ACID CHANGE IN WHOLE BLOOD*

By EDWIN P. LAUG

(From the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, May 19, 1934)

INTRODUCTION

Havard and Kerridge (1), while measuring the pH of whole blood at 38° with a modified bulb type glass electrode constructed of Woods' glass, observed a marked acid change, amounting on the average to 0.05 pH unit, which set in within 2 to 6 minutes after the blood had been drawn and placed within the electrode. The phenomenon occurred only in the presence of the corpuscles and was absent in plasma or serum. It was markedly slowed at room temperature and completely stopped at 0°. The usual antiglycolytic agent, NaF, was ineffective in preventing this change even in concentrations as high as 0.06 per cent, although Evans (2) had observed that 0.05 per cent was usually able to prevent a reduction in CO_2-combining power of blood kept at 38° for periods up to 30 minutes after drawing. All of their observations, except the rather anomalous ones on NaF, indicated that the change might perhaps be nothing other than a reflection of glycolysis. One feels, however, that this interpretation was not considered very seriously by them since they somewhat emphasized a "first acid change" as something sudden and distinct and not associated with the acid change of Evans; their pH-time curves indicated a well defined pH plateau lasting several minutes, and occurring between the two changes. Furthermore, a number of preliminary observations on lactic acid, while not entirely definite, failed to correlate this acid change with an increase in the level of lactic acid.

---

* Aided in part by a grant from the Special Faculty Research Committee of this University.

The possibility that the phenomenon could be an electric charge artifact, in some way connected with the settling out of the corpuscles, also seemed ruled out, since their set-up was so varied that the cells were made to settle either onto or away from the glass membrane without in any way modifying the change.

Some evidence of a decrease in the pH of blood at 38° immediately after removal from the body was obtained by the present author (3) with the hydrogen and quinhydrone electrodes by measuring the difference between the pH of plasma which had been in contact with cells at 38° for 10 to 12 minutes and plasma which had been separated from the cells immediately after drawing. The change averaged 0.03 pH, but it was not possible by this method to show how the change occurred, whether precipitously or slowly.

In view of the extremely important bearing these observations of Havard and Kerridge must have on practically all of the present data in the literature dealing with pH-CO_2-bicarbonate relationships in whole blood at 38°, it seemed desirable to reinvestigate the problem somewhat more fully.

Methods and Procedure

pH was measured at 38° with a glass electrode assembly fully described by Stadie *et al.* (4). It is, therefore, not necessary to go into a detailed description of the apparatus. It must be pointed out, however, that the active glass for the electrodes was Corning 015 instead of Woods' glass, and that the design of the electrode (the MacInnes (5) type) and the electrode pipette were entirely different from that described by Havard and Kerridge. Several modifications of Stadie's set-up were made: A stop-cock, fused to the top of the electrode pipette prevented possible loss of CO_2 from blood during lengthy determinations. All the glass parts, including the calomel and Ag·AgCl reference cells were enclosed in a stirred air bath, electrically maintained at 38°. The electrode pipette was provided with a jacket, kept filled with water, and covered with a layer of oil to prevent cooling by evaporation. The water was necessary in order to increase the heat capacity of the electrode chamber, so that when cold blood was placed within it, temperature equilibrium at 38° was reached within 1½ to 2 minutes by actual test. As an additional aid in rapid heat transfer, the walls of the electrode pipette were made quite thin. Several pipettes constructed without this water jacket were unsatisfactory, for in these, temperature equilibrium required about 10 minutes. Furthermore, well defined first acid changes proving to be nothing more than temperature artifacts traceable to this warming up, took place.

Blood was always transferred to the electrode pipette by positive pressure, never by suction. In order further to minimize effects due to CO_2 loss in transfer, the first 0.5 ml. of blood to enter the chamber was always forced up beyond the top stop-cock before this was closed. Between determinations, the pipette was flushed with normal saline in order to prevent possible precipitation of globulin films. Before and after each series of readings the glass electrode was standardized by phosphate buffer solutions of known pH. Checks to within 0.3 millivolt were required. Results on blood with electrodes beyond this limit of tolerance were discarded. With the glass electrodes used in this study, pH changes of 0.01 were significant.

Samples of human blood were obtained by venipuncture, with or without stasis, dog blood by heart puncture, and cat blood by carotid cannula. Bloods were stored in tonometers, over mercury, with the exception of a small number which were drawn directly into syringes. Since no change was observed to occcur in blood kept at 0°, most of the bloods were drawn directly into containers immersed in ice-salt mixtures, and then kept on ice until ready for use. When it was necessary to use blood without preliminary chilling, a rapid technique was developed which made it possible to transfer the blood from the body into the electrode pipette within an average time of 2½ minutes without any significant temperature or acid change occurring. As anticoagulant a 30 per cent solution of $K_2C_2O_4$ was routinely added to blood to make a 0.3 per cent solution. Such a comparatively high concentration was not found to interfere in any way with the acid changes observed as shown by controls in which either minimal amounts of oxalate were used (0.04 per cent) or where clotting occurred within the electrode pipette. The concentration of KF was varied to suit the experimental procedure.

Results

That whole blood becomes more acid at 38° immediately after removal from the body has been amply confirmed in this work on man, dog, and cat. In Fig. 1 are shown representative pH-time curves on human and dog blood at 38°. The solid circles represent blood which has been drawn by rapid technique transferred to the electrode within 2½ minutes without any appreciable change from body temperature. Confirming the experience of Havard

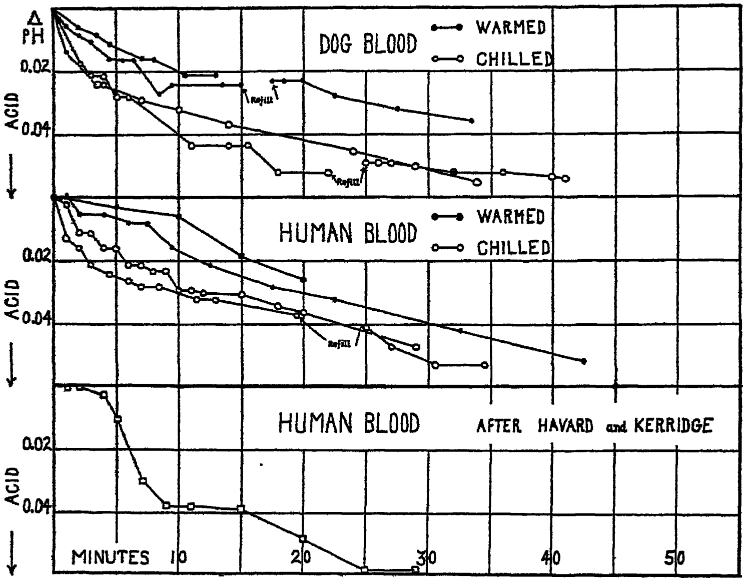


FIG. 1. The acid change in whole blood at 38°

and Kerridge that the change does not occur while blood remains cold, the clear circle curves represent such blood, chilled immediately after drawing and at some later time (several hours, sometimes) transferred to the electrode without prewarming. It will be noted that in the chilled blood the initial acid drift is somewhat steeper than in the prewarmed.

In Fig. 2 are reproduced curves from bloods which had reached an acid equilibrium by incubation for 3 to 6 hours at 38°. Here the acid change had manifestly become complete. The initial rapid change averaging about 0.01 pH unit, and complete within the first 2 minutes, is clearly due to warming up, since it is practically absent when the blood is first prewarmed. When allowance is made for this temperature effect, the apparent distinction between the initial drift rates of chilled and warmed bloods disappears.

For the sake of comparison, the lowest curve in Fig. 1 depicts the sequence of events as noted by Havard and Kerridge. The most important difference between their curve and the ones above is the extremely rapid change setting in after a short induction period and followed by a plateau lasting about 6 minutes. *This plateau seems to be the main justification of Havard and Kerridge for characterizing the phenomenon as a first acid change, entirely separate and distinct from the acid change taking place later and identified by most investigators as the result of glycolysis.*

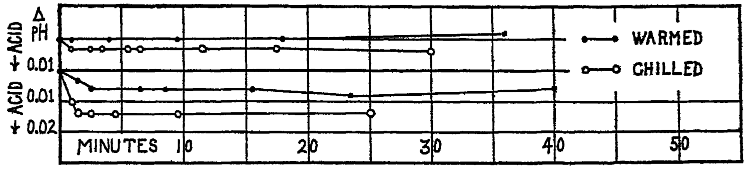


Fig. 2. Temperature control curves on blood after the acid change has taken place.

In this present work, none of the curves, whether on chilled or prewarmed blood, gives indication of any clearly defined and separate first acid change. The acid change observed, while increased in initial rate, merges without pause into those later changes already mentioned. The interpretation which can be placed on this evidence is simply that we are dealing with pH changes which are the reflection of an acid production, presumably glycolysis.

In Table I is given a compilation of the average pH changes observed in seventeen samples of human and twenty-four of dog blood during 5 minute intervals from the time the blood was put into the electrode. The average concentration of KF used was 0.1 per cent. It is clear that the maximum change occurs in the first two 5 minute periods and is practically double the change in the last two. It may be argued that because of the comparatively high fluoride and oxalate concentration (0.3 per cent $K_2C_2O_4$, 0.1

per cent NaF) the recorded changes are much less than those of Havard and Kerridge with 0.04 per cent $K_2C_2O_4$ and 0.06 per cent NaF. In disagreement with this is the fact that a number of bloods containing no fluoride at all did not show increased drifts. Bloods containing neither oxalate nor fluoride, and which, therefore, clotted in the electrode pipette, behaved similarly.

Platt and Dickinson (6), being unable to obtain any evidence for an acid change, have criticized the results of Havard and Kerridge on grounds that this sudden acid change represents a temperature artifact due to inadequate temperature control. They have shown that gradients of only 1° or 2° lead to large, apparent acid drifts. This criticism may be valid particularly since Havard and Kerridge used only air-jacketed apparatus, although it is difficult to understand why their curves should show an initial plateau of almost 4 minutes. The present experiments have shown satisfactorily that with water-jacketed equipment, there is no temperature artifact whatsoever with prewarmed blood, and only a small but predictable effect with chilled. The acid change observed is, therefore, not a temperature artifact.

TABLE I

Average Decrease in pH at 38° in Forty-One Samples of Whole Blood

Intervals in min.	0-5	5-10	10-15	15-20
pH decrease	0.008	0.007	0.004	0.004

There remains one other important control which has been repeated with practically every blood. As soon as a sample of blood was transferred to the electrode pipette, the remainder in the tonometer or syringe was immediately brought to 38° and kept in the air thermostat; under these circumstances, any acid change taking place in the pipette should also take place *pari pasu* in the tonometer. Thus the last reading on the first sample of blood in the pipette should agree with the first reading on a refill. That these "follow up" determinations did give such agreeing values is evident from the curves in Fig. 1. This is additional evidence that this acid change is a true one and not confined to the electrode pipette alone.

Effect of High Fluoride Concentration—The opinion is fairly well grounded in the literature that the fluoride ion is adequate for preventing glycolysis. The routine use of 0.05 to 0.1 per cent K or NaF is quite common. When it was observed that such concentrations were inadequate for preventing this sudden acid change in freshly drawn blood, it seemed not unreasonable to suppose that the increase in acidity was not associated with glycolysis. Most of the curves shown in Fig. 1 are on blood preserved with 0.3 per cent KF, three times the usual amount. It is apparent that even such comparatively high concentrations do not prevent the change.

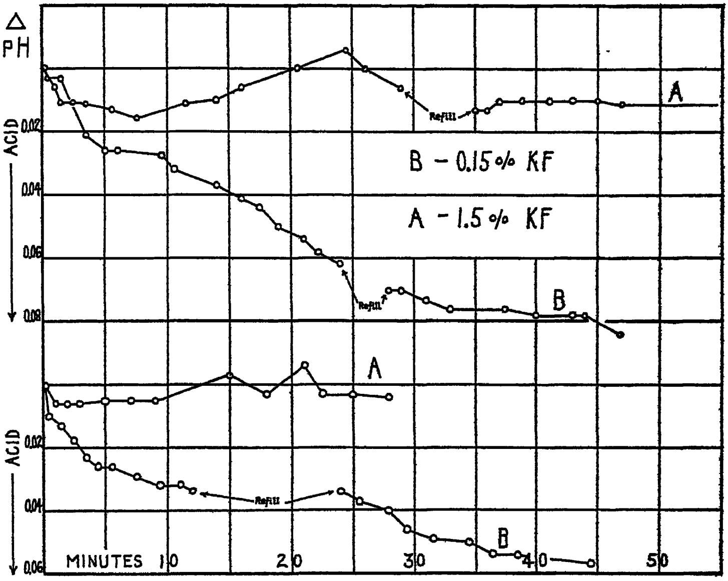


FIG. 3. The effect of high fluoride concentrations on the acid change

Nevertheless, it is conceivable that we are dealing with an extremely vigorous glycolysis. If this is so, then higher concentrations of fluoride might be necessary to stop it. Fig. 3 demonstrates that massive amounts of KF are effective in preventing this acid change completely in dog blood not only within the electrode pipette but also in a tonometer. In six samples of human blood preserved with 0.1 per cent NaF, the average change within the first 10 minutes was 0.013 pH unit more acid, while in four samples containing 1.0 per cent NaF, the change was only 0.004 pH more acid.

In view of the fact that fluorides are known to attack glass, it is possible that the lack of acid change may be due to changes in the glass electrode. Indications to the contrary, however, are: (1) Careful check of the electrode before and after contact with fluoride contained in blood and also buffers has demonstrated no changes; (2) "follow up" or refill determinations, such as shown in Fig. 3.

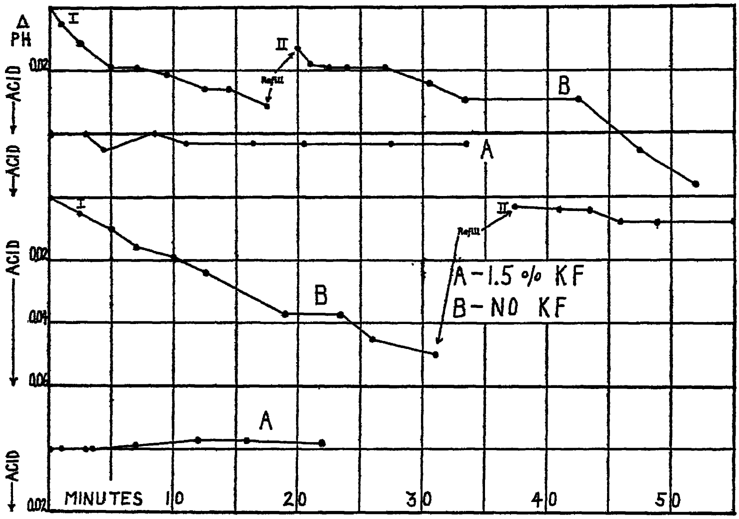


FIG. 4. The effect of high sedimentation rate of the corpuscles upon the acid change. Curves II are "follow up" curves of the experiments represented by the corresponding Curves I.

Sedimentation—When blood samples were placed in the electrode pipette, it was observed that the cells gradually settled out. In dog blood, sedimentation was more or less complete within 10 to 15 minutes. In the human blood examined the rate was even slower. In none of these experiments did there appear to be any correlation between the acid change and the sedimentation rate.

Cat blood, which has a high sedimentation rate, gave results indicating that extremely rapid settling produced an acid drift which is probably an artifact. Curves B in Fig. 4 show this.

In each case, the first values of the "follow up" curves, Curves II, do not agree with the last values of Curves I. This is offered as evidence that this acid change in Curves I is augmented by the sedimentation. That the usual acid change which was observed in all other cases takes place, perhaps somewhat more slowly in these cases, is shown by Curves II whose initial values both begin at slightly more acid values than for Curves I. An experiment with a glass electrode mounted in a pipette chamber which could be rotated in all planes showed that it made no difference whether the cells settled onto, away from, or past the glass membrane. The phenomenon is probably, therefore, not due to electric charging effects of the corpuscles. High concentrations of fluoride stop the settling, and of course also prevent any true acid change. This is shown in Curves A.

TABLE II

Changes in Lactic Acid and pH of Whole Blood at 38° Immediately after Removal from Body

	Subject	KF content	Initial		Incubation period at 38°	Final	
			pH	Lactic acid		pH	Lactic acid
		per cent		*mg. per cent*	*min.*		*mg. per cent*
Human blood (venous)	Sm.	0.0	7.42	13.2	23	7.41	10.6
	Ha.	0.0	7.30	5.2	23	7.29	7.7
	Wa.	0.15	7.35	5.6	22	7.33	12.2
	Sm.	0.12	7.34	15.0	26	7.33	17.3
			7.34	15.0	60	7.32	19.3
	Fe.	0.21	7.37	14.9	27	7.34	14.5
	Zo.	0.0	7.36	21.8	20	7.35	19.9
			7.36	21.8	60	7.33	25.4
Cat blood (arterial)	1	0.3	7.28	11.8	18	7.26	11.8
	2	0.03	7.38	17.6	20	7.35	17.3
	2	0.6	7.31	30.3	25	7.31	30.9
	3	2.0	7.34	5.1	28	7.31	8.2
	4*	0.0	7.01	173.3	21	6.98	174.5
	6	0.0	7.32	11.8	26	7.31	15.8
			7.32	11.8	63	7.27	18.3
	6	0.0	7.22	5.5	28	7.18	8.5

* Preparation from decapitate animal.

It cannot be decided from these experiments whether sedimentation is stopped by the antiglycolytic action or high electrolyte content. The latter appears the most probable.

Lactic Acid—If the acid change which has been observed is the result of glycolysis, it should be theoretically possible to demonstrate a rise of lactic acid. But in a closed system, such as a tonometer or electrode pipette, any CO_2 produced by the reaction between lactic acid and bicarbonate contributes to the increase in acidity because it cannot escape. Thus the buffer capacity of the blood toward lactic acid is apparently lowered. Under these conditions it has been calculated that an increase of the order of 2 mg. or less lactic acid per 100 ml. of blood is sufficient to produce a fall in pH of 0.01 unit. Such small increments of lactic acid offer practical difficulties in measurement. Lactic acid determinations for these studies were made in duplicate by the gasometric method of Avery and Hastings (7). Table II shows the results. In the shorter incubation periods, averaging 23 minutes, several bloods actually lost lactic acid although the pH change was uniformly toward the acid. In the longer periods, the rise in lactic acid was unmistakable. Except for the longer periods, the average of six determinations on human blood showed a rise of 1.1 mg. per cent of lactic acid and a fall of 0.015 pH unit; on seven cat bloods, a rise of 1.7 mg. per cent of lactic acid and a fall of 0.023 pH unit. The small number of determinations makes it impossible to attach any significance to the differences between man and cat. Taken by and large, it is believed that these results offer some evidence that the acid change which occurs in freshly shed blood can be classed as glycolysis.

SUMMARY

The phenomenon of a first acid change observed by Havard and Kerridge has been reinvestigated. A decrease in pH of whole blood at 38° immediately after removal from the body has been confirmed. The usual concentrations of KF (0.05 to 0.1 per cent) are ineffective in preventing the change; massive amounts of 1 to 2 per cent are completely effective. Evidence is presented to show that the decrease in pH may be due to lactic acid production. Temperature and corpuscle settling effects are discussed as possible artifacts.

The results seem to justify the interpretation that the first acid change is nothing more than glycolysis.

BIBLIOGRAPHY

1. Havard, R. E., and Kerridge, P. T., *Biochem. J.*, **23**, 600 (1929).
2. Evans, C. L., *J. Physiol.*, **56**, 146 (1922).
3. Laug, E. P., *J. Biol. Chem.*, **88**, 551 (1930).
4. Stadie, W. C., O'Brien, H., and Laug, E. P., *J. Biol. Chem.*, **91**, 243 (1931).
5. MacInnes, D. A., and Dole, M., *Ind. and Eng. Chem., Anal. Ed.*, **1**, 57 (1929).
6. Platt, B. S., and Dickinson, S., *Biochem. J.*, **27**, 1068 (1933).
7. Avery, B. F., and Hastings, A. B., *J. Biol. Chem.*, **94**, 273 (1931-32).

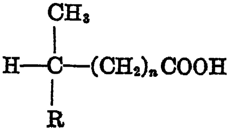
SYMMETRICAL DISUBSTITUTED METHANES PREPARED FROM MEMBERS OF OPTICALLY ACTIVE HOMOLOGOUS SERIES OF DISUBSTITUTED CARBOXYLIC ACIDS AND THEIR DERIVATIVES

By P. A. LEVENE and R. E. MARKER

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, May 23, 1934)

The present investigation was undertaken as a check on all previous work from this laboratory dealing with the problem of configurational relationship of the derivatives of disubstituted acids of the type



$$\mathrm{H}-\underset{\displaystyle \mathrm{R}}{\overset{\displaystyle \mathrm{CH_3}}{\mathrm{C}}}-(\mathrm{CH_2})_n\mathrm{COOH}$$

I*

in which R stands for any aliphatic radicle and $n = 0$ or any integer.[1] The starting materials were prepared either from malonic ester by double substitution with aliphatic halides, or from secondary aliphatic halides by condensation with malonic ester. Rearrangements may have occurred either in process of halogenation or during the reaction of substitution.

Again, the higher members were prepared from the lower by converting the functional group of the lower into a halide and then condensing the halide with malonic ester or with potassium cyanide,

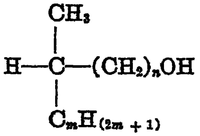
---

[*] Similar work on the homologous series containing an ethyl or a phenyl group is in progress. In the homologous series of the disubstituted propionic acids containing an ethyl group it would seem that the first member may rotate in the same direction as the higher members. For the present, this is merely an indication.

[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **103**, 299 (1933).

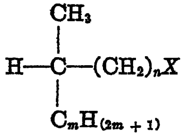
or through condensation of the Grignard reagent with formaldehyde or with carbon dioxide. Here also the possibility is not excluded that either in the process of halogenation or in the process of condensation a rearrangement took place.

There was reason to believe, on the basis of our previous experience, that such rearrangements do not occur in a degree sufficient to affect the validity of the conclusions published by us. The reasons are the following: First, the resolution of the synthetic *dl* substances into the active components requires numerous recrystallizations, the yield of the active material seldom exceeding 10 per cent of the starting material. This procedure should suffice to remove the bulk of the impurities. Second, in the carbinols of the type (II) the partial contribution of the OH group approaches



$$\begin{array}{c} CH_3 \\ | \\ H\text{—}C\text{—}(CH_2)_nOH \\ | \\ C_mH_{(2m+1)} \end{array}$$

II

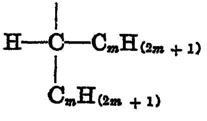


$$\begin{array}{c} CH_3 \\ | \\ H\text{—}C\text{—}(CH_2)_nX \\ | \\ C_mH_{(2m+1)} \end{array}$$

III

0 when $n = m$, and this phenomenon could occur only in cases when $m = n$. Third, the fact that in the series, regarded as homologous, of the type (III), where X stands for any functional group, the partial rotation of the group X and of that of the rest of the molecule was found to be of the same sign in all members, indicating that our arrangement of the substances into homologous series was correct.

Nevertheless, it was deemed desirable to test our conclusions by a more rigorous method. It was necessary to prove that in the substance of the general type (III), the carbon skeletons of the radicles $(CH_2)_nX$ and of $C_mH_{(2m+1)}$ are of the straight chain type. In order to do so, the chain C_n was made equal to C_m, and then the group or atom X was replaced by a hydrogen atom, thus leading to a substance of the general type



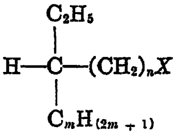
$$\begin{array}{c} CH_3 \\ | \\ H\text{—}C\text{—}C_mH_{(2m+1)} \\ | \\ C_mH_{(2m+1)} \end{array}$$

IV

These substances would be symmetrical only when both radicles $C_mH_{(2m+1)}$ were normal.

Two groups of substances of the type (III) were selected; in one, $m = 3$, in the other, $m = 4$. In both cases, the hydrocarbons were inactive when n was made equal to m. In other words, the conclusions formulated previously regarding the configuration of the substances of the general type (II) are correct. If rearrangements took place in the course of any of these reactions, then the products of such rearrangements were removed in course of resolution or of purification of the other derivatives, thus showing that they were present, if at all, in small proportions.

Work in the same direction on the group of substances of the general type of



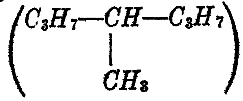
$$\begin{array}{c} C_2H_5 \\ | \\ H—C—(CH_2)_nX \\ | \\ C_mH_{(2m+1)} \end{array}$$

is now in progress and the results will be published in the near future.

Incidentally, occasion was taken to hydrogenate the levo-2,8-heptenol-(4) ($C_3H_5CHOH—CH = CHCH_3$). In this case, propyl bromide was converted into the Grignard reagent and then condensed with butyraldehyde. The saturated carbinol was found inactive and therefore had the constitution of di-*n*-propylcarbinol. Thus, it was shown that if any rearrangement took place in any phase of the reaction leading to the above unsaturated carbinol, it took place in a small measure only, so that the main product could be obtained in pure state by recrystallization either of the half phthalic ester directly, or by recrystallization of some crystalline salt of the half phthalic ester. In cases in which complicated fractionation columns are not accessible, this method may serve for purification of synthetic secondary carbinols.

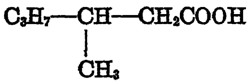
EXPERIMENTAL

Symmetrical Dipropylmethylmethane (4 Methyloctane) from Levo-Methylpropylpropionic Acid

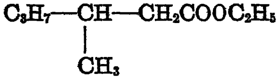


$$\left(\begin{array}{c} C_3H_7—CH—C_3H_7 \\ | \\ CH_3 \end{array}\right)$$

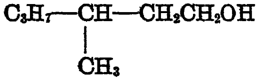
—The hydrocarbon was prepared by means of the following synthesis. Methyl-*n*-propylcarbinol was converted into 2-bromopentane by gaseous hydrogen bromide. This was condensed with malonic ester and the resulting propionic acid resolved by crystallizing its cinchonidine salt from 50 per cent alcohol. This was not resolved to the maximum. This, in turn, was converted into the following derivatives by methods previously described, giving the molecular rotations as follows:



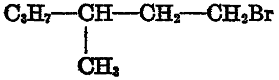
$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2COOH$ $[M]_D^{25} = -2.43°$



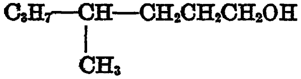
$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2COOC_2H_5$ $[M]_D^{25} = -0.70°$



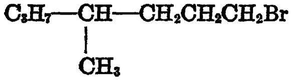
$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2CH_2OH$ $[M]_D^{25} = -1.41°$



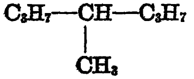
$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2Br$ $[M]_D^{25} = +13.41°$



$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2CH_2CH_2OH$ $[M]_D^{25} = 0°$



$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-CH_2CH_2CH_2Br$ $[M]_D^{25} = +5.79°$

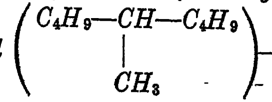


$C_3H_7-\underset{\underset{CH_3}{|}}{CH}-C_3H_7$ $[M]_D^{25} = 0°$

50 gm. of 1-bromo-4-methylheptane, $[M]_D^{25} = +5.79°$, were converted into the Grignard reagent by magnesium in dry ether. This was poured on ice. The hydrocarbon was purified by shaking with cold concentrated sulfuric acid, sodium carbonate solution, and finally refluxed with sodium until it tarnished it no more. B.p. 117.5°. Yield 12 gm. $\alpha = 0$.

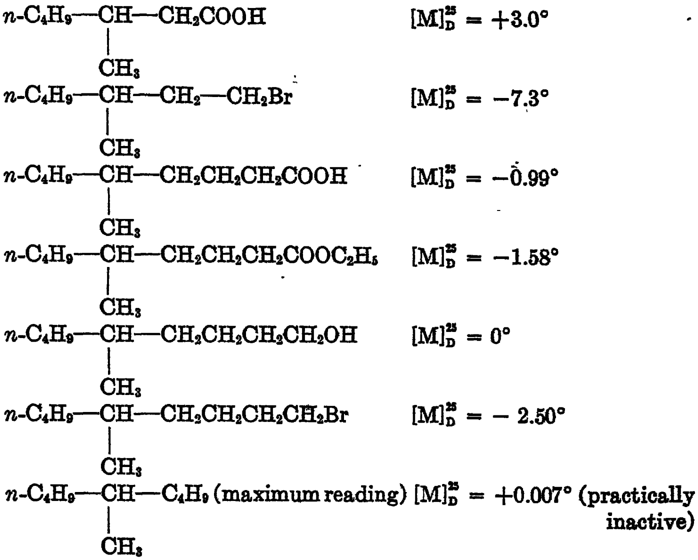
2.950 mg. substance : 9.090 mg. CO_2 and 4.190 mg. H_2O
C_8H_{18}. Calculated. C 84.1, H 15.9
Found. " 84.0, " 15.9

Methyl-Di-n-Butylmethane (5-Methylnonane) from Methylbutylpropionic Acid



$$\left(\begin{array}{c} C_4H_9\text{—}CH\text{—}C_4H_9 \\ | \\ CH_3 \end{array}\right)$$

—Methyl-di-*n*-butylmethane was prepared from methylbutylpropionic acid through the following compounds which were previously described.



$$\begin{array}{ll} n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2COOH & [M]_D^{25} = +3.0^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2\text{—}CH_2Br & [M]_D^{25} = -7.3^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2CH_2CH_2COOH & [M]_D^{25} = -0.99^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2CH_2CH_2COOC_2H_5 & [M]_D^{25} = -1.58^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2CH_2CH_2CH_2OH & [M]_D^{25} = 0^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}CH_2CH_2CH_2CH_2Br & [M]_D^{25} = -\ 2.50^\circ \\ n\text{-}C_4H_9\text{—}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{—}C_4H_9\ \text{(maximum reading)} & [M]_D^{25} = +0.007^\circ\ \text{(practically inactive)} \end{array}$$

50 gm. of 1-bromo-5-methylnonane, $[M]_D^{25} = -2.50^\circ$, were reduced by means of the Grignard reagent, as described for dipropylmethylmethane. The hydrocarbon was purified by the usual methods. B.p. 162–163°. Yield 20 gm. $\alpha = +0.007^\circ$.

3.116 mg. substance : 9.650 mg. CO_2 and 4.375 mg. H_2O

$C_{10}H_{22}$.	Calculated.	C 84.4, H 15.6
	Found.	" 84.4, " 15.7

Di-n-Propylcarbinol (n-C_3H_7—$CHOH$—C_3H_7) *from l-2,3-Heptenol-(4)*—Inactive 2,3-heptenol-(4) (C_3H_7—CHOH CH = CH—CH_3) was prepared by means of the Grignard reagent on butyraldehyde. This was converted into the half phthalic ester and resolved by crystallizing its brucine salt from acetone. The phthalate had the following rotation.

$$[\alpha]_D^{25} = \frac{-0.25^\circ \times 100}{1 \times 20 \times 1} = -1.25^\circ \text{ (in benzene)}$$

The phthalate was converted into the carbinol by steam-distilling an alkaline solution of the phthalate. B.p. 157°, 760 mm. *D* 25/4 = 0.832.

$$[\alpha]_D^{25} = \frac{-0.58^\circ}{1 \times 0.832} = -0.70^\circ; [M]_D^{25} = -0.80^\circ$$

3.390 mg. substance: 9.120 mg. CO_2 and 3.735 mg. H_2O

$C_7H_{14}O$. Calculated. C 73.6, H 12.4
Found. " 73.4, " 12.3

The above carbinol was reduced by shaking with hydrogen in the presence of platinum oxide. The reduced carbinol was distilled. B.p. 152–153°, 760 mm. $\alpha = 0$.

3.195 mg. substance : 8.495 mg. CO_2 and 3.980 mg. H_2O

$C_7H_{16}O$. Calculated. C 72.4, H13.9
Found. " 72.5, " 13.9

GLYOXALASE

II. THE DISTRIBUTION OF GLYOXALASE IN TISSUES OF NORMAL AND CANCEROUS ALBINO RATS

By MURIEL E. PLATT and E. F. SCHROEDER

(From the Cancer Research Laboratories, University of Pennsylvania Graduate School of Medicine, Philadelphia)

(Received for publication, May 9, 1934)

In a recent communication (1) conditions were defined for the determination of the glyoxalase activity of acetone-yeast by means of a manometric method. In principle, the method depends upon the measurement of the amount of CO_2 evolved in the neutralization, by sodium bicarbonate, of the lactic acid produced from methylglyoxal by the action of the enzyme. This method has now been applied to a study of the distribution of the enzyme in various tissues of normal and cancerous albino rats.

The kinetic behavior of glyoxalase in aqueous extracts of animal organs (with the exception of kidney and pancreas, in which the results are complicated by the presence of an inhibitor) is identical with that observed in the case of acetone-yeast suspensions. This identity extends to the specific activating effect of glutathione, the constancy of reaction rate, the effect of methylglyoxal concentration, and the relation between quantity of enzyme and lactic acid formed in unit time.

The question of the quantitative distribution of glyoxalase in tissues is of interest in connection with the possible relation of this enzyme to the glycolysis process. If glyoxalase is directly concerned in the production of lactic acid from carbohydrates, then one would expect those tissues which have high glycolytic activity, such as cancer, to contain large amounts of the enzyme as compared to tissues with low glycolytic acitvity. A number of investigators (2–4) have presented data on the distribution of glyoxalase in tissues. These data, however, have little significance, since no effort was made to control the glutathione concentration. As was shown in our previous paper (1) quantitative comparisons can be made only if the concentration of glutathione is held constant. In the process of extracting the enzymes, variable amounts of glutathione are also extracted. Furthermore, as was shown by Hopkins and Elliott (5), tissue suspensions differ widely in their ability to keep glutathione in the reduced form essential for enzyme activation. Therefore during the extraction, and subsequent preparation of the reaction mixtures, considerable changes in the glutathione concentration always occur. We have been able to overcome this difficulty by oxygenating the aqueous extracts at 0° until the reduced glutathione was completely removed. Constant amounts of the latter were then added for the determination of enzyme content.

By this method no evident relationship was found to exist between glyoxalase content and glycolytic activity. Extracts of tumor tissue contain about the same amount of glyoxalase as leg muscle. The average values found for the various tissues are as follows: liver, 100; tumor, 9; leg muscle, 10; diaphragm, 10; spleen, 24; kidney, 5; pancreas, 8. The livers of animals with Walker No. 256 carcinoma tended to be low in glyoxalase, while those of animals with Philadelphia No. 1 sarcoma were in the normal range. Beyond this, no marked differences were found between corresponding organs of normal and cancerous animals.

A similar conclusion was reached in experiments on the glyoxalase activity of slices of intact tissue, without added glutathione. The average values found are as follows: liver, 100; tumor, 25; diaphragm (muscle), 30; spleen, 55; pancreas, 49; kidney, 123. The values for tumor tissue are lower than for any of the other tissues, all of which have a much lower anaerobic glycolysis. Again, no differences of significance were found between corresponding organs of normal and cancerous animals.

The glyoxalase activity of kidney extracts is not constant, but falls off very rapidly during the determination. Slices from this organ, on the other hand, exhibit a constant, high reaction rate. Pancreas shows a similar, though less pronounced behavior. From this it may be concluded that kidney contains an inhibitory substance liberated during the extraction, which is probably similar to the pancreatic 'anti-glyoxalase' already known (2).

EXPERIMENTAL

Preliminary Studies on Animal Tissue Glyoxalase

Method—Except for the preparation of the enzyme solutions, the procedure for determining glyoxalase activity was identical in all respects with that previously described for acetone-yeast.

For the preliminary studies on the properties of the enzyme, aqueous extracts of rat liver, muscle, and tumor were used. The tissue was thoroughly ground up with sand in a mortar, treated with 5 parts by weight of distilled water, and allowed to stand for 30 minutes at room temperature with frequent stirring. After centrifuging, the supernatant liquid was placed in a large test-tube in an ice bath, and a slow stream of oxygen bubbled through for 2 hours. A blank determination of the enzyme activity without added glutathione was then made. If the CO_2 evolution was insignificant (less than 5 c.mm. in 20 minutes for amounts of extract up to 1 cc.), it was assumed that all the GSH had been oxidized. In most cases, the latter is completely removed by this treatment, although the oxygenation may be continued for at least 5 hours without damage to the enzyme itself. When extracts thus prepared are kept in the ice box, practically no loss of enzyme activity occurs for at least 48 hours, as shown by complete reactivation on addition of glutathione.

The CO_2 blank described above, which is probably due to reaction between protein and methylglyoxal, may usually be neglected in determining enzyme activity; or it may be corrected for by subtraction. As with acetone-yeast, it is essential, in a series of comparative experiments, to adopt a uniform order in adding the various reagents.

Kinetics—Since the kinetic behavior of glyoxalase from liver, muscle, and tumor was found to be identical in all essential respects with that previously reported for acetone-yeast, complete data on the points investigated are not presented. Table I shows the similarity in the activating effect of increasing amounts of glutathione on constant amounts of glyoxalase from various sources. The amount of enzyme used was that which gave approximately 65 c.mm. of CO_2 in 20 minutes with 0.25 mg. of glutathione. The reaction mixtures contained, in addition, 2 mg. of methylglyoxal and 0.4 cc. of sodium bicarbonate (0.2 M) in a total volume of 2 cc. The temperature was 25°. The close agreement in the values obtained with different amounts of glutathione indicates the identity of the enzyme from the various sources.

The glyoxalase in tissue extracts appears to be somewhat more stable than that in acetone-yeast suspensions, due perhaps to the smaller amount of cell residue present. This is shown by the fact that the rate of the enzyme reaction is constant for at least 1 hour both at 25° and at 37°, while acetone-yeast glyoxalase, which has a constant rate at 25°, decreases in activity almost from the start at the higher temperature. This constancy in reaction rate for tissue glyoxalase is maintained even in the presence of large amounts of glutathione and methylglyoxal (up to 5 and 8 mg. respectively) while under the same conditions acetone-yeast glyoxalase is slightly inhibited. As with acetone-yeast, the rate is independent of the methylglyoxal concentration (1 to 5 mg. in 2 cc.) when low concentrations of glutathione are used (below 0.5 mg.), but increases with increasing substrate concentration when larger amounts of glutathione are present (2 mg.).

TABLE I

Effect of Increasing Amounts of Glutathione on Activity of Glyoxalase from Various Sources

GSH added	CO_2 in 20 min.			
	Acetone-yeast	Liver	Muscle	Tumor
mg.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
0.1	20	22		
0.2	37	38	35	36
0.3	45	48		
0.5	64	65	65	66
0.75	77	78		
1.0	88	86	94	90
2.0		91		
5.0	78	78		

For the determination of the glyoxalase activity of tissue extracts, the same standard conditions employed in the work on acetone-yeast were adopted; namely, 0.25 mg. of glutathione, 2 mg. of methylglyoxal, and 0.4 cc. of bicarbonate (0.2 M), in a total volume of 2 cc., and a temperature of 25°. Under these conditions, direct proportionality exists between the quantity of enzyme used and the amount of CO_2 evolved in 20 minutes, as shown in Table II.

Relation between Loss of Glyoxalase Activity and Glutathione—Freshly prepared extracts from different organs, as well as from the same organ from different animals of the same species, vary widely in the rate at which they lose their glyoxalase activity. Occasionally rat liver extracts were found to be completely inactivated by the time extraction was finished. In other cases, several hours oxygenation was necessary to produce the same result. Rabbit liver extracts generally were much more stable, although if the animal had previously been fasted, extracts were obtained which decreased very rapidly in activity. Laked blood was particularly stable, retaining a large part of its glyoxalase activity for several days at 0°. Human blood was more stable than rat blood.

TABLE II

Relation between Quantity of Enzyme and CO_2 Formation at 25°

Liver extract	CO_2 in 20 min.	Liver extract	CO_2 in 20 min.
cc.	*c.mm.*	*cc.*	*c.mm.*
0.1	34	0.6	200
0.2	70	0.7	234
0.3	104	0.8	272
0.4	136	0.9	304
0.5	170	1.0	340

Fig. 1 shows that these rapid losses in activity are due to disappearance of reduced glutathione. Freshly prepared rat liver extract (unoxygenated) was allowed to stand at room temperature. At intervals, the glyoxalase activity was determined without added glutathione. At the same time, the glutathione content of the extract was determined by the iodate procedure of Woodward and Fry (6), the protein being removed by adding 2 cc. of extract to 16 cc. of water and 2 cc. of 22 per cent sulfosalicylic acid. Iodometric methods for glutathione are not wholly satisfactory in the case of tissues, particularly when incubations are involved. Nevertheless, the agreement between loss of activity and loss of glutathione is fairly close, the slight spread between the curves with time indicating probably the formation of other iodine-consuming substances. After 3 hours, addition of the original amount of glutathione (as determined by titration) almost completely restored the activity, showing that the enzyme was still present. It should be pointed out that losses in glutathione apparently do not occur during the actual enzyme determination, since constant reaction rates continue for long periods of time (except for kidney and pancreas extracts). This is probably due either to union with the substrate, or to the protective action of the anaerobic conditions employed.

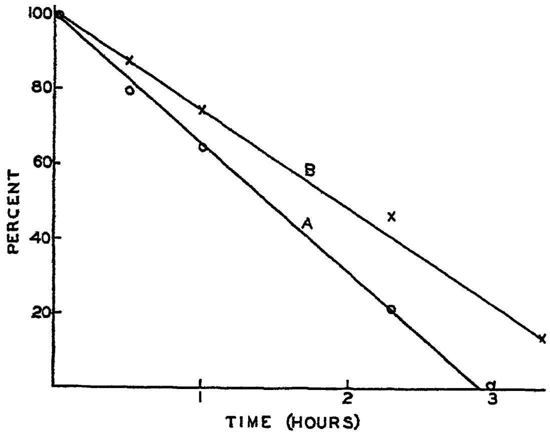


FIG. 1. Relation between loss of glyoxalase activity and loss of glutathione in rat liver extract. Curve A, glyoxalase activity; Curve B, glutathione.

Distribution of Glyoxalase in Tissues

Extraction of Enzyme—In order to determine the most suitable procedure for extracting the enzyme from tissues, the following experiment was carried out. Equal weights of fresh pig liver were treated as follows: the tissue was (*a*) ground with sand, extracted for 1 hour with 5 parts by weight of water, and centrifuged; (*b*) minced in a grinder, extracted for 1 hour with 5 parts of water, and centrifuged; (*c*) frozen in liquid nitrogen, crushed, extracted 1 hour with 5 parts of water, and centrifuged; (*d*) minced, and suspended in 5 parts of water; (*e*) dried with acetone and ether, and suspended in 5 parts of water (calculated on a basis of wet weight).

The various extracts and suspensions were oxygenated to remove glutathione. The activities were then measured by the standard procedure, with equal amounts (0.4 cc.) of the various preparations. The volumes of CO_2 evolved in 20 minutes were 80, 78, 72, 88, and 97 c.mm. respectively. As was to be expected, the suspensions gave slightly higher activities than the extracts. The use of suspensions, however, proved unsatisfactory owing to the impossibility of obtaining them fine enough for the accurate measurement of the small volumes involved. Also it was more difficult to remove glutathione from the suspensions, probably because of the presence of reducing mechanisms. The activities found for the various extracts agree satisfactorily, and consequently in the distribution studies the method of grinding with sand was used, as described in the previous section. It was found that extending the time of extraction beyond 30 minutes does not increase the amount of enzyme in solution.

Glyoxalase in Extracts—Table III shows the results obtained with tissues of well fed normal and cancerous albino rats; a number of figures are included for tissues of white mice. Depending on the glyoxalase content, from 0.2 to 1.0 cc. of oxygenated 1:5 extract was used for the determination. The results are expressed as c.mm. of CO_2 obtained in 10 minutes from 0.4 cc. of extract, assuming that direct proportionality exists between quantity of enzyme and amount of CO_2 formed.

With the exception of liver, no evident differences exist between cancerous and normal animals in the glyoxalase content of corresponding organs. Rats and mice with carcinomas tended to have a low liver glyoxalase, while rats with sarcomas were near the normal range. The low values obtained for cancerous tissue indicate that no relation exists between the high glycolytic activity of this tissue and its glyoxalase content. Muscle, liver, and spleen are much less active glycolytically, although the first contains as much, and the latter two much more glyoxalase than cancer. A number of determinations on diaphragm, not included in Table III, gave approximately the same values as leg muscle.

These results differ to some extent from those reported by previous workers, owing to their failure to control the glutathione concentration. Sakuma (4) found that mammalian muscle, spleen, and kidney contain about 40 per cent as much glyoxalase as liver.

He also states that young sarcoma tissue contains as much as liver. Ariyama (3) likewise reports that in white rats muscle and kidney are about 40 per cent, and spleen 30 per cent as active as liver.

In view of our experiments, it is difficult to understand the high glyoxalase content found by these workers in kidney. Kidney and pancreas extracts, even with added glutathione, were either practically inactive at the start, or rapidly decreased in activity during the determination, while extracts from the other organs maintained a constant reaction rate. This indicates the presence of inhibiting substances in pancreas and kidney. Consequently the results given in Table III are not truly representative of the glyoxalase content of these two organs.

TABLE III

Glyoxalase Content of Tissue Extracts

Animal	CO_2 in 10 min. from 0.4 cc. extract					
	Liver	Muscle (leg)	Tumor	Spleen	Kidney	Pancreas
	c.mm.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
Rat (normal)	238	25		74	9	17
“ “	243	23		51	12	
“ “	241	21		45	11	16
“ “	213	17				17
“ “	195	14		46	10	
“ (sarcoma, Philadelphia No. 1)*	141	22	15	45	11	15
“ (“ “ “ 1)*	223	20	18	44		
“ (“ “ “ 1)*	231	18	16	48		18
“ (“ “ “ 1)*	195	19	19		10	
“ (carcinoma, Walker No. 256)*	91	20	16	46	9	14
“ (“ “ “ 256)*	131	21	16	48	10	
Mouse (normal)	193	23				
“ “	207	14				
“ (Ehrlich carcinoma)	120	24	23			
“ “ “	107	21	20			

* Firm, rapidly growing tumors, with little necrosis, were used. For a description of the tumors, see Waldschmidt-Leitz, McDonald, and coworkers (7).

Glyoxalase in Tissue Slices—In order to determine the actual glyoxalase activity (as distinguished from glyoxalase content) existing in tissues, a number of determinations were made with thin slices of tissue. The rats were killed by a blow on the head, bled from the neck, and the organs removed and sliced with a razor. Leg muscles were cut transversely; since this cuts through the cells, the results obtained are probably not representative. As an example of muscular tissue, therefore, diaphragm was included. Only the outside, non-necrotic portion of tumors was used. For the activity determination, the reaction mixtures contained 1.2 cc. of Ringer's solution, 0.4 cc. of bicarbonate, and 2 mg. of methylglyoxal, in a total volume of 2 cc. No glutathione was added. Blank determinations without methylglyoxal were insignificant except in the case of leg muscle, which had a very low glyoxalase activity, and they were therefore neglected. After the determination, the tissue was dried at 110° for 1 hour, and weighed.

With slices, including pancreas and kidney, the rate of glyoxalase action was constant for at least 1 hour, and directly proportional to the weight of tissue used, provided the slices were not too thick.[1]

---

[1] After completion of this investigation, Jowett and Quastel (8) published the results of a similar study on the glyoxalase activity of tissue slices. They found that the reaction rate decreased quite rapidly from the start of the experiment, which must be ascribed to the higher temperatures they employed (37°), since all other conditions were essentially identical with ours. They also report that the glyoxalase activity is not proportional to the tissue weight, but to the tissue area, and increases as the thickness of the slice is decreased. This is believed to be due to incomplete diffusion of substrate into the tissue. Although we have made no measurements of tissue thickness, merely cutting the slices as thinly as possible, we have had no difficulty in obtaining reproducible results on the same tissue (within 10 per cent). Jowett and Quastel state that, "If the intrinsic activity falls, the methylglyoxal will penetrate further into the slice (no change in diffusion coefficient being supposed to take place), and as a result the observed change in activity will not be so great as the change in intrinsic activity." It is conceivable that a similar explanation accounts for our constant results. At the lower temperature employed in our experiments, the intrinsic activity of the tissue will be considerably lowered, and as a result the substrate will be able to penetrate farther into the interior portions of the slice. Consequently the rate will be less dependent upon the thickness of the slice. In agreement with our results, Jowett and Quastel find a higher glyoxalase activity for kidney than for liver slices. However, their high values for Jensen rat sarcoma (about the same as for guinea pig liver) are not in agreement with our results for tumors.

With thick slices the rate was less than would be expected from the weight of tissue used, probably due to incomplete diffusion of the substrate into the interior cells. When slices were allowed to stand in Ringer's solution for long periods of time, the glyoxalase activity fell off, probably due to loss of glutathione. For example, liver slices in 4 hours lost about 20 per cent of their activity. When proper precautions were observed, duplicate determinations on the same tissue agreed within 10 per cent.

TABLE IV

Glyoxalase Activity of Rat Tissue Slices

	CO_2 in 20 min. per mg. dried tissue						
	Liver	Tumor	Kidney	Muscle (leg)	Spleen	Diaphragm	Pancreas
	c.mm.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
Normal	7.6		10.9	0.85	6.3	3.5	
"	12.0		12.9	0.58			6.0
"	13.3		20.4	1.18	6.2	2.3	
Sarcoma (Philadelphia No. 1)	10.1	2.2	14.2		6.5		7.0
" (" " 1)	11.9	3.2		0.45		3.5	
" (" " 1)	13.2	2.4	13.2	0.73	7.6		5.0
Carcinoma (Walker No. 256)	10.7	3.0		0.60	6.1	3.9	
" (" " 256)	7.1	3.8	14.0				5.1
" (" " 256)	17.2	3.3	13.4	0.58	5.2	4.0	4.2
Average for slices	100	25	123	6	55	30	49
" " extracts	100	9	5	10	24	10	8

The results are shown in Table IV. A computation is included of the average glyoxalase values obtained from all rat tissue extracts and slices examined, when a value of 100 is assigned to liver. As with extracts, slices from corresponding organs of normal and cancerous animals showed no significant variation in activity, even livers from animals with carcinoma being normal. Tumor slices showed about the same activity as diaphragm, although leg muscles were much less active. The low values obtained with the latter are undoubtedly due to diffusion of glutathione out of the injured cells. Evidence in support of this is seen in the fact that addition of 0.25 mg. of glutathione increases the activity by 400 per cent, while the same amount added to the other tissues produces only a 20 to 40 per cent increase. Warburg, Posener, and Negelein (9) reported that slices of rat carcinoma tissue were as active in converting methylgloxal to lactic acid as liver tissue. Our experiments do not support this finding.

The experiments with slices also lead to the conclusion that no relation exists between glyoxalase activity and glycolytic activity. Representative values for the anaerobic glycolysis of the various tissues studied are as follows (c.mm. of CO_2 formed in 1 hour per mg. of dried tissue): liver, 3.3; tumor (Jensen sarcoma and Flexner carcinoma), 25 to 40; kidney, 3.7; muscle (diaphragm), 6.1; spleen, 8.3; pancreas, 3.5. The glyoxalase activity of tumor slices is lower than for any of these types of tissue, while the glycolytic activity is from 3 to 12 times as great.

Further evidence for the existence in pancreas and kidney extracts of an inhibitor of glyoxalase is obtained by comparing the low activity of the extracts with the high activity of the slices. This is especially marked in the case of kidney. The fact that the reaction rate does not decrease with time when slices are used indicates that the inhibitor does not exert its effect until it has been extracted. While the presence of an antiglyoxalase in pancreas has been known for a long time, the occurrence of a similar substance in kidney has not previously been reported. It appears to be present in much larger amounts in kidney than in pancreas, and thus far we have found it in kidneys of rat, rabbit, and pig. Further work on the nature of the inhibitor is in progress.

SUMMARY

1. The kinetic behavior of animal tissue glyoxalase has been shown to be identical with that previously reported for acetone-yeast glyoxalase.

2. A quantitative study of the distribution of glyoxalase in aqueous extracts of organs of normal and cancerous rats and mice was carried out. No evident relationship was found between the high glycolytic activity of cancer tissue and its glyoxalase content. A similar conclusion was reached in a study of the glyoxalase activity of slices of intact tissue.

3. Evidence has been presented for the occurrence in kidney

tissue of an inhibitor of glyoxalase, similar to the antiglyoxalase of pancreas.

BIBLIOGRAPHY

1. Platt, M. E., and Schroeder, E. F., *J. Biol. Chem.*, **104**, 281 (1934).
2. Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, **15**, 463 (1913).
3. Ariyama, N., *J. Biol. Chem.*, **77**, 359 (1928).
4. Sakuma, F., *J. Biochem.*, Japan, **12**, 247 (1930).
5. Hopkins, F. G., and Elliott, K. A. C., *Proc. Roy. Soc. London, Series B*, **109**, 58 (1931).
6. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, **97**, 465 (1932).
7. Waldschmidt-Leitz, E., McDonald, E., and coworkers, *Z. physiol. Chem.*, **219**, 115 (1933).
8. Jowett, M., and Quastel, J. H., *Biochem. J.*, **28**, 162 (1934).
9. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, **152**, 309 (1924).

GASOMETRIC MICRODETERMINATION OF PHOSPHORIC ACID

By ESBEN KIRK

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 18, 1934)

Phosphoric acid is precipitated as strychnine phosphomolybdate, the precipitate is washed, redissolved in acetone, the acetone is evaporated, and the carbon content of the residue determined gasometrically by wet combustion by the micromethod of Van Slyke, Page, and Kirk (11).

For precipitation of phosphoric acid by strychnine molybdate the procedure followed is essentially that given by Embden (1) and Roche (8), with some added refinements necessary in analysis of the minute amounts to which the gasometric method is applied (0.005 to 0.02 mg. of phosphorus). It appears that attempts to determine phosphoric acid by titration (Myrbäck (4) and Roche (8)) or by weighing (Myrbäck (4)) of the strychnine phosphomolybdate precipitate have not been entirely satisfactory in analysis of samples containing less than 0.02 mg. of phosphorus. This limit is given by Roche as the lower one to which microtitration is applicable, and Myrbäck in analyses of standard phosphate solutions by the microgravimetric procedure reports deviations from the theoretical as large as 6 to 14 per cent for samples containing 0.006 to 0.016 mg. of phosphorus.

The gasometric method here described has the same applications to blood, urine, tissues, and organic phosphorus compounds as the gravimetric method of Embden.

Reagents

For Phosphorus Determination

Embden's strychnine molybdate reagent. This is prepared as described by Embden (1) and by Tisdall (9). The reagent should not be prepared fresh before each analysis, as a considerable precipitate forms in the first 24 hours after mixing of the strychnine nitrate solution and the molybdate solution. After this period of time the mixed reagent is filtered through acid-washed filter paper. The strychnine molybdate reagent can be used for at least 4 months, although slight formation of precipitate continues to take place. The reagent when used should be absolutely clear; it is convenient to filter a small portion of the stock solution immediately before use.[1]

0.04 per cent aqueous filtered solution of phenol red.

Merck's Blue Label 28 per cent ammonia water.

Approximately 1 N hydrochloric acid.

Dilute nitric acid. 1 cc. of concentrated nitric acid, tested for freedom from phosphoric acid, is diluted to 100 cc. with distilled water.

Acetone, distilled.

For Precipitation of Blood or Plasma Proteins

10 per cent solution of phosphorus-free trichloroacetic acid. The trichloroacetic acid must be purified by distillation, as all commercial samples examined contained so much phosphorus as to interfere greatly with the analysis of the small amounts of phosphorus to which the gasometric method is applied. The distillation is performed *in vacuo*, the distilling flask containing the trichloroacetic acid being immersed in an oil bath of a temperature of about 130°. The purified acid is preserved in paraffin-sealed glass-stoppered bottles. A 10 per cent solution is prepared as described by Wakefield and Power (12) by melting the solid acid in a water bath at 60–65° and transferring 5 cc. to water by means of a previously warmed pipette, and diluting the solution with water to 80 cc. in a measuring cylinder.

---

[1] In most publications, except that of Tisdall (9), the fresh preparation of the mixed strychnine molybdate reagent before each analysis is emphasized. This feature might be a necessary one in the original application of the strychnine molybdate precipitation of phosphoric acid to direct colorimetric determination of the turbidity of the unflocculated precipitate by Pouget and Chouchak (6), as the reagent turns yellow in less than 1 hour after mixing of the components. In other analyses, however, it would, as mentioned above, appear to be a disadvantage to prepare the mixed reagent immediately before use. The yellow color of the reagent is unimportant in analyses in which direct colorimetry is not employed.

For Digestion of Organic Phosphorus Compounds

Sulfuric-nitric acid digestion mixture. Equal volumes of highest purity sulfuric and nitric acids, tested for freedom from phosphoric acids.

Concentrated nitric acid, phosphorus-free.

Procedure

In all analyses the sample is neutralized to phenol red, made up to 6 cc. volume, and 2 cc. of Embden's strychnine molybdate reagent added. The precipitate is transferred to an asbestos filter and washed with 1 per cent nitric acid. The washed precipitate is redissolved in acetone, in which it was found to be readily soluble. The acetone solution is transferred by suction to a combustion tube, the acetone evaporated, and the carbon of the strychnine is determined by the manometric combustion method. The phosphorus content is calculated from the carbon value. The carbon content which we have found in this precipitate indicates 3.5 molecules of strychnine and 73.5 atoms of carbon per atom of phosphorus, so that minute amounts of phosphorus can be easily determined.

The essential difference in the preparation of the sample from the procedure described by Embden, besides the use of a filtered strychnine molybdate reagent, has been the washing of the precipitate with 1 per cent nitric acid instead of washing with ice-cold 1:5 diluted reagent followed by ice water. This substitution was found necessary, because it was regularly noticed that a turbidity developed directly under the asbestos filter during the subsequent washings with distilled water. This turbidity is caused by precipitation of the reagent itself, and occurs when the dilute strychnine molybdate reagent is further diluted with water. The use of ice-cold washing solutions was not found necessary.

Preparation of Blood or Plasma Filtrate—1 cc. of whole blood, plasma, or serum is measured into a 10 cc. volumetric flask containing 5 cc. of 10 per cent trichloroacetic acid. The flask is filled to the mark with water, and, after about 15 minutes, the contents are filtered through a small acid-washed filter paper. In analyses of normal blood or plasma 3 cc. of the filtrate are used for each determination.

In analysis of whole blood or cells the usual precautions for preventing hydrolysis of acid-soluble organic phosphorus compounds must be observed (5).

Preparation of Urine Filtrate—2 cc. of urine, well mixed to suspend any insoluble phosphates, are measured into a 100 cc. volumetric flask containing 10 cc. of a 10 per cent solution of trichloroacetic acid. The acid serves to dissolve any precipitated phosphate and to precipitate any protein present. After 10 minutes the flask is filled to the mark with water and, if necessary, the solution is filtered. 1 to 5 cc. of the filtrate, representing 0.02 cc. of urine per cc. of the solution, are measured into a centrifuge tube.

Neutralization of Trichloroacetic Acid Blood or Urine Filtrate—To the filtrate in a 15 cc. centrifuge tube, calibrated at 6 cc. volume, is added 1 drop of the 0.04 per cent phenol red indicator solution, followed by 1 drop of strong ammonia water. The ammonia addition causes the color of the solution to change from yellow to red. 1 N hydrochloric acid is now added drop by drop until the color changes back to yellow, after which the solution is made up to 6 cc. volume with water.

Precipitation of Strychnine Phosphomolybdate—2 cc. of the strychnine molydate reagent are added rapidly to the 6 cc. of solution. After the addition the contents of the tube are thoroughly mixed. This can be best accomplished by rotating the tube around its axis while the tube is held in an oblique position. The tube is permitted to stand for 20 to 45 minutes to complete the precipitation, and the process of mixing is repeated once or twice during this period. 20 minutes suffice for complete precipitation of the maximum amounts of phosphorus which can be determined in the manometric gas apparatus, while longer precipitation times than 1 hour may cause slight resolution of the precipitate. If the contents are mixed as described above, a flocculent precipitate forms within a few minutes and settles quickly to the bottom.

Transfer of Strychnine Phosphomolybdate Precipitate to Asbestos Filter and Washing of Precipitate—For transfer and washing of the strychnine phosphomolybdate precipitate an apparatus similar to that described by Pregl ((7) Figs. 23-b, 24, pp. 110, 120) for transfer and washing of halogen silver precipitates is used (see Fig. 1). The ground glass stopper sealed to the filter tube fits both the vacuum flask and the combustion tubes. The filter tube contains a lower layer of well packed coarse asbestos and an upper layer of fine asbestos (see Fig. 1). This asbestos filter tube was found much

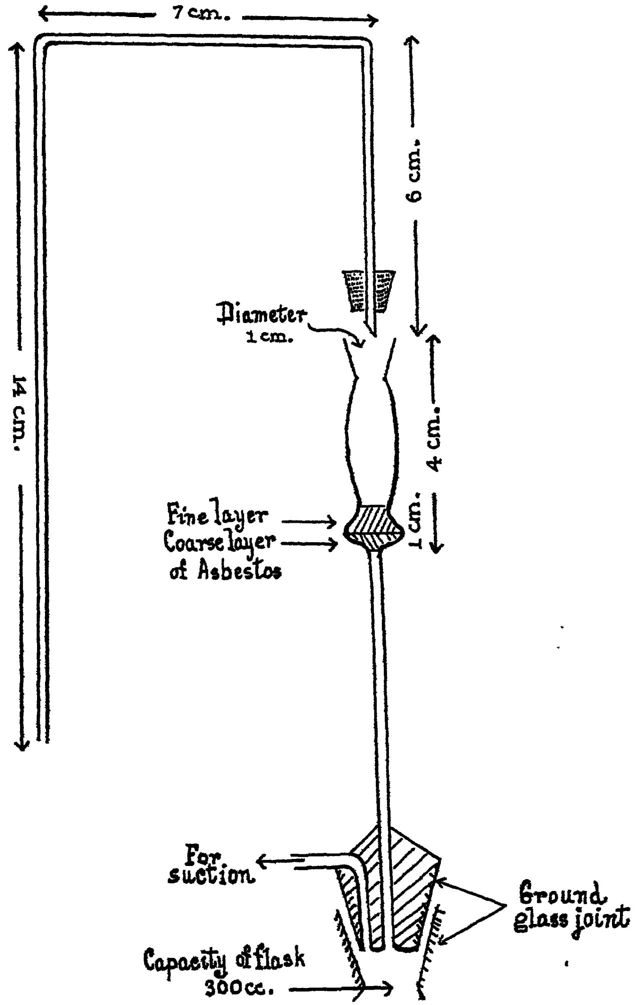


FIG. 1. Apparatus for transfer and washing of strychnine phosphomolybdate precipitate. Obtainable from Eimer and Amend, New York.

more practical than various kinds of sintered glass filters tried. The filtration could be more rapidly accomplished through the asbestos mat than through glass filters of a porosity sufficiently fine to retain the precipitate. A single asbestos packing may serve for about 200 filtrations. Rather strong vacuum suction can be applied without danger of loss of material. The transfer of the 8 cc. of fluid requires about 2 minutes. The tip of the transferring tube is first brought into contact with the precipitate at the bottom of the tube, so that the supernatant, nearly clear, fluid may serve as washing solution. Any remaining particles of precipitate in the centrifuge tube are transferred by means of three 0.5 cc. samples of dilute 1 per cent nitric acid, the third portion of washing fluid being used for washing the outside of the immersed part of the transferring tube. Two more 0.5 cc. portions of nitric acid are afterwards added directly to the cup of the filter tube, care being taken to wash the walls of the cup. These last washings are filtered through rather slowly and are followed by two 0.5 cc. washing samples of distilled water, likewise added directly to the filter cup. The last washings should be color-free.

Dissolving of Precipitate and Transfer of Sample to Combustion Tube—The filter tube is removed from the suction flask, the outside of the capillary under the stem is washed once with water, and the stem is inserted into the ground neck of the combustion tube. 0.7 cc. of redistilled acetone is pipetted onto the precipitate in the filter tube, and the precipitate and the upper, fine portion of the asbestos are stirred up with a small pointed glass rod. The precipitate dissolves rapidly and the acetone is transferred by suction to the combustion tube. Two similar acetone additions of 0.5 cc. each are made, the asbestos and precipitate each time being stirred up thoroughly. After the third portion of acetone has been passed through the filter, 0.5 cc. of distilled water is added and the upper layer of asbestos is again stirred up; this aqueous acetone causes solution of a small fraction of the precipitate which does not dissolve in absolute acetone. After two more 0.5 cc. acetone samples have been sucked through the asbestos mat, the outside of the tube below the ground glass stopper is washed with a few drops of acetone. The last washing sample passes through the asbestos filter free of color. The total time involved in transferring, washing, and dissolving the strychnine molybdate precipitate is approximately 10 minutes.

Evaporation of Acetone—The combustion tube is immersed 1 inch into hot water in a beaker on a steam bath, and the sample is evaporated to dryness. As the sample already contains water, further addition for complete removal of the organic solvent is not necessary. After cooling, the tube is ready for combustion.

Combustion—2.5 minutes suffice for complete combustion.

TABLE I

For Calculation of Phosphorus from P_{CO_2} Obtained at 2 Cc. Volume after Wet Combustion of Strychnine Phosphomolybdate Precipitate

Temperature	Factors by which mm. P_{CO_2} are multiplied to give mg. phosphorus in sample analyzed
°C.	
15	0.00005190
16	5162
17	5134
18	5106
19	5078
20	5053
21	5025
22	5000
23	0.00004972
24	4947
25	4923
26	4899
27	4877
28	4852
29	4831
30	4806
31	4785
32	4764
33	4746
34	4725

Blank—A blank is run with each series of analyses, distilled water being used instead of the phosphoric acid sample. This blank includes both the *c* correction of the gasometric carbon analysis and a blank caused by the minute amounts of phosphoric acid in the reagents. The CO_2 pressure from phosphoric acid impurities gave a reading of about 8 mm. at the 2 cc. mark with the reagents used. The blank from such impurities has been quite constant.

Calculation—Mg. phosphorus in sample = mg. carbon found/28.4.

The phosphorus content of the sample may be calculated directly from the factors given in Table I. These factors are obtained by dividing the factors for calculation of carbon by 28.4.

Cleaning of Filter Tube—The filter tube requires no cleaning with cleaning mixture. 1 cc. of water is passed through the asbestos mat after each analysis to remove traces of acetone.

Average Error—The error of the analysis is usually of the magnitude of ±0.5 per cent.

Determination of Phosphorus in Organic Compounds

Digestion of Organic Matter—This is done by Neumann's wet ashing method as described by Van Slyke, Hiller, and Berthelsen (10). 0.2 cc. of whole blood or 0.5 cc. of plasma is pipetted into a 200 × 15 mm. Pyrex tube which has been previously calibrated at 25 cc. Each tube is constricted to 10 to 12 mm. in diameter at the calibration mark and is identical with the tubes used for digestion in the manometric total base analysis. 1 cc. of the sulfuric-nitric acid digestion mixture is added, together with a Pyrex glass bead to prevent bumping. The tube is heated with a microburner until a dark brown color appears. It is then removed from the flame and, while hot, more nitric acid is added, a drop at a time, and the digestion continued. This process is repeated two or three times until the liquid is perfectly clear. The heating is continued until white fumes appear and then for 10 minutes longer. Just enough heat is applied to keep the tube filled with white fumes, but not enough to drive them out of the tube. Extra addition of sulfuric acid is unnecessary and should be avoided to prevent a too great concentration of ammonium sulfate in the diluted and neutralized digest, as concentrated solutions of ammonium sulfate may in themselves cause precipitation of the strychnine molybdate reagent.

Neutralization of the Digest—To the cooled digest are added 5 cc. of distilled water and 1 drop of the phenol red solution.[2] Concentrated ammonia water is then added drop by drop. The solution in the presence of much strong acid is first rose-colored. As ammonia is added it assumes the usual yellow color of dilute acid solutions, and when excess ammonia is present the alkaline red color of the indicator appears. Enough 1 N hydrochloric acid is added drop by drop to turn the solution acid to phenol red. The contents of the tube are again cooled and afterwards made up to 25 cc. volume. After thorough mixing, a 6 cc. sample is measured out for precipitation.

---

[2] Phenol red is preferred as indicator to brom-phenol blue in neutralization of the acid digest. It was found that brom-phenol blue when added to the strongly acid digest might split off free bromine, which would subsequently cause precipitation of the strychnine molybdate reagent

TABLE II

Analysis of Standard Phosphate Solutions

Phosphorus in sample	Carbon found	Phosphorus found calculated as mg. carbon/28.4	Per cent deviation from theoretical
mg.	*mg.*	*mg.*	
0.0100	0.2822	0.009937	−0.63
0.0100	0.2838	0.009993	−0.07
0.0100	0.2820	0.009930	−0.70
0.0100	0.2830	0.009965	−0.35
0.0100	0.2846	0.010021	+0.21
0.0100	0.2852	0.010042	+0.42
0.0150	0.4228	0.014887	−0.75
0.0150	0.4229	0.014891	−0.73
0.0200	0.5680	0.020000	±0.00
0.0200	0.5700	0.020070	+0.35
0.0200	0.5664	0.019944	−0.28
0.0200	0.5720	0.020141	+0.70

Results

The results of analyses of standard phosphate solutions are given in Table II. Two different standard solutions (Merck's and Kahlbaum's monobasic potassium phosphate) and strychnine molybdate solutions were used. The recovery of phosphate added to normal plasma is given in Table III. In Table IV results obtained by the gasometric method in analyses of normal urines are compared with gravimetric phosphorus determinations by the method of Mathison (3) in the modification of MacKay and Butler (2). The gasometric results are slightly higher than the gravimetric,

TABLE III

Recovery by Gasometric Method of Phosphate Added to Human Plasma

	Plasma in sample	Phosphorus in sample found	Phosphorus	Average phosphorus
	cc.	*mg.*	*mg. per cent*	*mg. per cent*
Original plasma	0.3	0.01178	3.927	
	0.3	0.01175	3.917	3.922
After addition of 3.334 mg. phosphate phosphorus per 100 cc. plasma	0.2	0.01453	7.265	
	0.2	0.01466	7.330	7.297
Recovered, 3.375 mg. per cent phosphorus or 101.2 per cent				

TABLE IV

Comparison of Gasometric Phosphate Phosphorus Determination in Urine with Gravimetric Determination by Method of Mathison in Modification of MacKay and Butler (2)

Urine No.	Method	Urine or phosphate solution in sample	Phosphorus in sample	Phosphorus
		cc.	*mg.*	*mg. per cent*
1	Gasometric	0.02	0.01440	72.00
	"	0.02	0.01443	72.15
	Gravimetric	50.00	35.58	71.16
	"	50.00	35.74	71.48
2	Gasometric	0.02	0.01266	63.30
	Gravimetric	50.00	31.18	62.36
	"	50.00	31.04	62.07
3	Gasometric	0.02	0.01263	63.15
	"	0.02	0.01277	63.85
	Gravimetric	50.00	31.18	62.36
	"	50.00	31.15	62.30
4	Gasometric	0.02	0.01649	82.45
	"	0.02	0.01651	82.55
	Gravimetric	50.00	40.23	80.46
	"	50.00	40.76	81.52
Standard phosphate solution containing 1 mg. phosphorus, per cc.	Gravimetric	50.00	49.07	98.14
	"	50.00	48.93	97.86

but probably represent the truer values, as gravimetric analyses of standard phosphate solutions by the same procedure constantly gave results lower than the theoretical.

SUMMARY

Phosphoric acid is determined by precipitation with strychnine molybdate and wet combustion of the precipitate by the gasometric method of Van Slyke, Page, and Kirk (11). The phosphorus content is calculated from the carbon content of the sample. The method is applicable to analyses of samples containing 0.005 to 0.02 mg. of phosphorus. With samples of 0.01 to 0.02 mg. of phosphorus, the average error is ±0.5 per cent.

BIBLIOGRAPHY

1. Embden, G., *Z. physiol. Chem.*, **113**, 138 (1921).
2. MacKay, E. M., and Butler, A. M., in Peters and Van Slyke (5), p. 858.
3. Mathison, G. C., *Biochem. J.*, **4**, 233 (1909).
4. Myrbäck, E., *Z. physiol. Chem.*, **148**, 197 (1925).
5. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **2**, 872 (1932).
6. Pouget, L., and Chouchak, D., *Bull. Soc. chim.*, **5**, 104 (1909); **9**, 649 (1911).
7. Pregl, F., Die quantitative organische Mikroanalyse, Berlin (1917).
8. Roche, J., *Bull. Soc. chim. biol.*, **10**, 1061 (1928).
9. Tisdall, F. F., *J. Biol. Chem.*, **50**, 329 (1922).
10. Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, **74**, 659 (1927).
11. Van Slyke, D. D., Page, I. H., and Kirk, E., *J. Biol. Chem.*, **102**, 635 (1933).
12. Wakefield, E. G., and Power, M. H., in Piersol, G. N., *et al.*, The cyclopedia of medicine, Philadelphia, **6**, 571 (1932).

GASOMETRIC MICRODETERMINATION OF LIPIDS IN PLASMA, BLOOD CELLS, AND TISSUES

BY ESBEN KIRK, IRVINE H. PAGE, AND DONALD D. VAN SLYKE

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 18, 1934)

Outline of Analyses

The lipids are extracted from plasma with alcohol-ether mixture by Bloor's (1914) method as somewhat modified by Man and Gildea (1932–33). A portion of the alcohol-ether extract is saponified and used for *total cholesterol* precipitation by Windaus' (1909) digitonin method. The digitonide precipitate is measured by its carbon content, which is determined by the manometric microcombustion method of Van Slyke, Page, and Kirk (1933).

In estimation of the other lipid fractions the alcohol-ether extract is freed of non-lipid substances by driving off the solvent at a temperature not exceeding 60° and redissolving the lipids in the residue with petroleum ether. Aliquots of the petroleum ether solution are analyzed as follows: The *total lipids* are estimated by determination of the total non-volatile carbon. The *free cholesterol* is determined by digitonin precipitation without saponification. *Lipid amino nitrogen* (of *cephalin* and perhaps other amino lipids) is estimated by driving off the solvent, emulsifying the residue in water, and determining the aliphatic amino nitrogen by the micromanometric nitrous acid procedure of Van Slyke (1929). The *total phosphatides* are estimated from the phosphorus content of the lipid mixture. For this purpose the organic matter is destroyed and the phosphoric acid in the residue is determined by the procedure of Kirk (1934), in which strychnine phosphomolybdate is precipitated and its amount is estimated by manometric determination of the carbon in the precipitate. We have found this procedure more exact than methods based on precipitation of the phosphatides with acetone plus magnesium chloride (Bloor, 1929) or calcium chloride (Katsura *et al.*, 1933). Like Boyd (1931), we have been unable to find conditions for quantitative precipitation by these procedures of such minute amounts of phosphatides as we have desired to determine. We have, however, been able to obtain nearly quantitative results by precipitating larger samples of phosphatides, such as were employed by Bloor (1929). A full discussion of the acetone-$MgCl_2$ precipitation of the phosphatides will be given later, together with a procedure for gasometric estimation of the precipitated phosphatides.

In the carbon combustions by the method of Van Slyke, Page, and Kirk (1933), a combustion period of 2.5 minutes was found to be sufficient for all determinations described in this paper. Blanks must be run on the volatile organic solvents in which the lipid samples are dissolved. 10 cc. of solvent should not leave enough organic non-volatile residue to give a measurable pressure at 2 cc. volume.

All pieces of apparatus which come into contact with the lipid material are initially cleaned in chromic-sulfuric acid. If the combustion tube at the end of each analysis is immediately protected from contamination with dust, rinsing with distilled water suffices to clean the tube for the next analysis. The detached tube may conveniently be placed in a 400 cc. beaker, which is covered by a watch-glass or a crystallizing dish or may be kept inverted in a metal rack stand in contact with a clean towel. The filter tube, used for washing the strychnine phosphomolybdate precipitate, is washed with water after each analysis to remove traces of acetone. It requires no cleaning with chromic-sulfuric acid.

The small amount of material (0.2 to 0.6 mg. of carbon) required for carbon determination, exact to 1 part in 200, makes it possible to obtain accurate results for total lipids with the extract from 0.05 cc. of blood, and for cholesterol with the extract from 0.15 cc. 3 cc. of plasma suffice for determination of all the lipid fractions mentioned above, with duplicate analyses in each case.

Reagents for Lipid Extraction

Alcohol-ether 3:1. 3 volumes of redistilled 95 per cent alcohol and 1 volume of redistilled ether, as used by Bloor.

Petroleum ether. Commercial petroleum ether which distils at between 30–60° is washed by shaking with concentrated sulfuric acid (200 cc. of sulfuric acid for 1500 cc. of petroleum ether). This can be conveniently done in large glass-stoppered bottles. The petroleum ether should be left in contact with the sulfuric acid for at least 2 days, during which period the bottle is frequently shaken. The petroleum ether is then decanted off and distilled.

Sand, purified from organic material by ignition.

Fat-free filter paper (extracted with ether).

Reagents for Cholesterol Saponification and Precipitation

Saturated solution of sodium hydroxide in 95 per cent alcohol. This solution is kept in a glass bottle, stoppered by a vaseline-greased glass stopper (to prevent precipitation of carbonates). The bottle is kept in the dark. The solution should be discarded if it turns yellow.

0.04 per cent aqueous filtered solution of phenol red.

Approximately 1 N hydrochloric acid.

1 per cent solution of Merck's digitonin in 50 per cent redistilled alcohol. In preparing this reagent it is usually necessary to heat the solution slightly.

Ether, redistilled.

Reagents for Lipid Nitrogen

For micro-Kjeldahl digestion. To 10 volumes of the 3:1 sulfuric-phosphoric acid mixture of Van Slyke (1926–27) is added 1 volume of a 1 per cent solution of metallic selenium in concentrated sulfuric acid. The selenium catalyzes the digestion of the otherwise somewhat resistant fatty acids.

For lipid amino nitrogen. The reagents are those for the manometric amino nitrogen method described by Van Slyke (1929) and by Peters and Van Slyke (1932).

Reagents for Lipoid Phosphorus

The reagents are described in the accompanying paper by Kirk (1934).

Special Apparatus Required

Apparatus for Microdetermination of Total Lipids Only

Two Jena sintered glass filters, No. 4-G-4, 2 cm. in diameter, with ground joints.

Two 25 cc. volumetric flasks with ground joints to fit the sintered glass filters.

Apparatus for Complete Lipid Analyses

For Extraction of Lipids

Two 100 cc. volumetric flasks with ground joints.

Two water-cooled Graham condensers with ground joints to fit 100 cc. flasks.

For Preparation of Petroleum Ether Extract

Several 1000 cc. Pyrex beakers.

Two Jena sintered glass filters, No. 17-G-4, 7 cm. in diameter, with ground joints (Fig. 5).

Two 100 cc. volumetric suction flasks with ground joints to fit the sintered glass filters.

For Storing of Petroleum Ether Extract

Several 125 cc. glass-stoppered bottles.

For Estimation of Total Lipid, Cholesterol, and Phosphorus (Strychnine Molybdate) by Carbon Combustion

Several combustion tubes (described by Van Slyke, Page, and Kirk (1933)).

For Saponification and Estimation of Total Cholesterol

Several ordinary Pyrex test-tubes 175 × 25 mm.

One transfer tube sealed into glass stopper (Fig. 1).

One 25 cc. volumetric flask with ground joint to fit transfer tube stopper.

For Washing of Cholesterol Digitonide

Several filter sticks with detachable alundum filter disks (Fig. 3).

For Digestion, Precipitation, and Washing of Lipoid Phosphorus

Several 200 × 25 mm. Pyrex digestion tubes with constriction at the 25 cc. mark.

Several standard *Pyrex* glass beads (ordinary glass does not suffice).

Several 15 cc. centrifuge tubes for precipitation.

One Pregl asbestos filter tube with ground joint (Fig. 1, Kirk, 1934).

One 250 cc. Erlenmeyer flask with ground joint.

For Lipid Total Nitrogen—Several 250 × 25 mm. Pyrex digestion tubes for micro-Kjeldahl digestion.

For Lipid Amino Nitrogen—Several 150 cc. beakers.

One *Van Slyke-Neill manometric apparatus* serves for the final determination of all fractions.

Preparation of Plasma Extracts

Drawing Blood for Analysis—Heparin is used instead of oxalate as anticoagulant because oxalate may combine with some of the phosphatides (MacLean and MacLean, 1927). According to Shope (1928) both oxalate and citrate may cause partial saponification of the cholesterol esters. Man and Gildea (1932–33) also found distinctly lower values for total fatty acids in oxalated plasma than in heparinized plasma and serum. Heparinized plasma is preferred to serum, because of the possibility that cephalin might be influenced by the process of coagulation. Furthermore, the use of plasma permits the immediate separation of cells, so that little time is given for phosphorus compounds of the cells to enter the plasma. To determine whether heparin offered the same protection as citrate against disintegration of the platelets (and consequent contamination of the plasma by cephalin contained in the platelets), the lipid amino nitrogen content of a heparinized plasma sample was compared with that of a citrated sample of the same plasma. Practically identical values were found in the two samples. Heparin is also preferred to citrate as anticoagulant in order to avoid changes in the water content of the plasma due to the salt effect: in the comparative analysis of the heparinized and citrated plasma mentioned above 13 per cent higher values for total fat were found in the heparinized than in the citrated sample.

When a complete analysis including all the lipid fractions described below is to be made, enough blood is drawn to yield 3 cc. of clear plasma. This will provide material for duplicate analyses. If only a part of the fractions described is to be determined, the blood sample may be correspondingly diminished. The volumes of normal plasma represented in the amounts of ether-alcohol extract used for single determinations are as follows: total lipids, 0.045 cc.; total cholesterol, 0.06 cc. (the equivalent of 0.15 cc. is used for saponification); free cholesterol, 0.1125 cc.; total phosphatides, 0.054 cc. (the equivalent of 0.225 cc. of plasma is used for each digestion); total lipid nitrogen, 0.225 cc.; lipid amino nitrogen, 0.45 cc.

Separation of Cells and Plasma—As soon as possible after it is drawn, the blood sample is centrifuged in a 15 cc. centrifuge tube. 45 minutes centrifugation at 2000 R.P.M. are sufficient to leave in suspension in the plasma less than 1 per cent of the original number of platelets. The separation of platelets from the plasma must be complete or too high values for plasma lipid amino nitrogen will result. Also, in pipetting off the supernatant plasma care is taken to prevent elements from the buffy layer over the red cells from contaminating the sample.

Extraction of Lipids from Plasma

For the complete analysis 3 cc. of plasma are added drop by drop to 75 cc. of redistilled alcohol-ether mixture (3:1) in a volumetric flask, which is calibrated at 100 cc. volume and provided with a neck ground to fit a water-cooled condenser. During the addition of the plasma the mixture is agitated thoroughly to obtain a finely divided precipitate. Two clean glass beads are added to produce even boiling. The alcohol and ether are then allowed to reflux for 1 hour. After this period the condenser is rinsed twice with alcohol-ether and the flask is cooled to room temperature. The content is made up to volume with alcohol-ether, and, after thorough mixing, is quickly filtered through a fat-free filter paper into a glass-stoppered clean bottle. Approximately 90 cc. of filtrate are obtained. This filtrate, each cc. of which represents 0.03 cc. of plasma, will be termed A-E Extract A.

If only total lipids are to be determined, 0.2 cc. of plasma may be extracted with 10 cc. of alcohol-ether in a 25 cc. volumetric flask and made up to 25 cc. for filtration. The extraction is most conveniently made by immersing the volumetric flask into a beaker with boiling water and keeping the flask immersed for a few seconds after the contents have started boiling. The addition of a glass bead prevents bumping. The filtrate, each cc. of which represents 0.008 cc. of plasma, will be termed A-E Extract B. Values for total plasma lipids obtained by this microprocedure agree within 2 per cent with values obtained by analysis of A-E Extract A.

Extraction of Lipids from Blood Corpuscles

After centrifugation of the blood sample and syphoning off of the plasma, the buffy coat and the upper 3 mm. of the compact layer of red blood cells are removed by suction. By means of a pipette with a wide opening 3 to 3.5 cc. of cells are transferred to a 20 cc. beaker which has been previously weighed to within 1 mg. As much of the sample is used as will leave the pipette without difficulty; the exact size of the sample is estimated by renewed weighing of the beaker. 3 cc. of sand, purified from organic material by ignition, are added to the cells in the beaker and the whole is thoroughly mixed by stirring with a glass rod. A semiliquid mixture results which can easily be poured into the extraction flask with the help of the stirring rod. The small amount of residue left in the beaker is transferred to the extraction flask with two successive portions of 4 cc. of water, the water also being used to rinse the glass rod. When the transfer is completed, the contents of the extraction flask are mixed by vigorous shaking to insure complete laking of the cells. The alcohol-ether extraction fluid is then added slowly with frequent shaking. A glass stopper is inserted and the contents of the flask again vigorously shaken for half a minute to insure a finely divided precipitate, after which the mixture is boiled under a reflux condenser.

After the reflux extraction is completed, the contents are as usual made up to 100 cc. volume, after which an extra 3 cc. of the alcohol-ether mixture is measured into the flask to compensate for the 3 cc. of sand used.

Extraction of Lipids from Tissues

The instructions given by Bloor (1929, p. 278) for weighing and grinding of the sample are followed. 3 cc. of ignited sand are mixed with the sample. 300 mg. of liver, kidney, pancreas, or heart are used for reflux extraction with 75 cc. of alcohol-ether, making a final volume of 103 cc.

Total Cholesterol in Alcohol-Ether Plasma Extract

Saponification of Cholesterol Esters—5 cc. of A-E Extract A are pipetted into a Pyrex test-tube (175 × 22 mm.) and 2 cc. of a saturated solution of sodium hydroxide in redistilled 95 per cent alcohol are added. The test-tube is partly immersed for 2½ hours in water at 85°, or, more conveniently, placed in an electric oven at 85°. When the contents of the tube have been reduced to about 3 cc., a cork stopper covered with tin-foil is firmly inserted into the opening of the tube. The stopper is provided with a small furrow to allow escape of alcohol vapors.

The total cholesterol could also be determined in the petroleum ether solution of purified lipids, instead of in the total alcohol-ether extract, but there are advantages in using the latter. It permits one to begin saponification as soon as the plasma is extracted. In consequence the saponified mixture for total cholesterol is ready for digitonin precipitation at about the same time that the Petrol Extract A (see below) is ready for free cholesterol precipitation, so that both cholesterol determinations can then be carried through together. Also, the alcohol-ether extract mixes homogeneously with the alcoholic sodium hydroxide, whereas petroleum ether does not; and the conditions of saponification are more readily controlled with the alcohol-ether extract. The exact control of cholesterol ester saponification has required more care than any other step of the analysis. If treatment with alkali is not severe enough, which is likely to occur if the temperature during the saponification falls much below 85°, saponification is incomplete and the total cholesterol yield is low. But if the treatment is too severe, some cholesterol is destroyed and the results are again too low. The latter may occur if the volume of the saponification mixture becomes too much reduced towards the end of the saponification. This is more difficult to avoid if the petroleum ether solution is used.

Neutralization of Alkali—After $2\frac{1}{2}$ hours the tube is cooled and 2 drops of a 0.04 per cent aqueous phenol red solution added. The contents of the tube are then neutralized with 1 N hydrochloric acid.

Extraction of Cholesterol with Petroleum Ether—From the saponified solution the total cholesterol is extracted by means of five successive 5 cc. portions of petroleum ether, and is transferred to a 25 cc. calibrated volumetric flask. The contents of the tube are carefully heated with each portion of petroleum ether by immersion in a beaker of hot water, and agitated to insure thorough mixing. Part of the petroleum ether evaporates; the rest is drawn as completely as possible into the 25 cc. flask, as shown in Fig. 1. Care must be taken to prevent drops of the water-alcohol solution from being drawn over into the volumetric flask. So much petroleum ether evaporates that the five portions do not fill the 25 cc. flask.

After rinsing the capillary of the transferring tube below the ground stem with petroleum ether, the contents of the flask are made up to 25 cc. with petroleum ether. A clean ground glass stopper is inserted into the flask and the petroleum ether solution is mixed. If the plasma is normal, a 10 cc. sample of petroleum ether, representing 0.06 cc. of plasma, is pipetted into a combustion tube for digitonin precipitation. If the plasma is lipemic, only 5 cc. of the petroleum ether solution are used, representing 0.03 cc. of plasma.

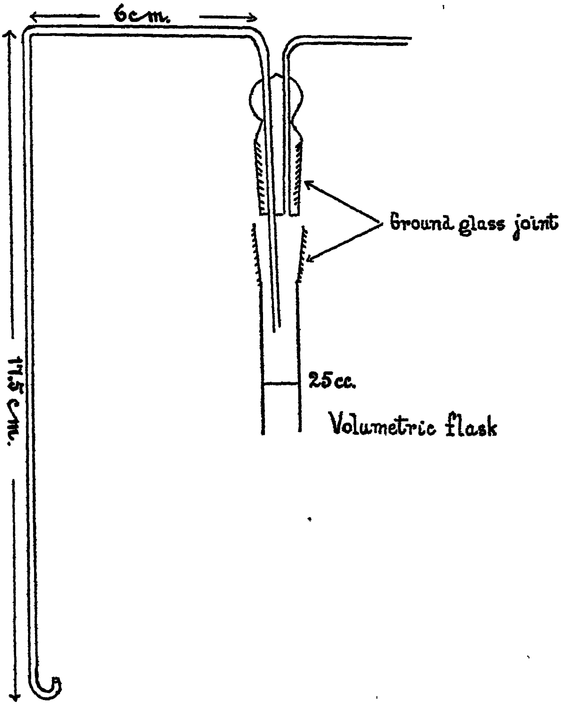


Fig. 1. Arrangement for drawing lipid extract into volumetric flask.

Note on Washing of Volumetric Flask and Pipette—The volumetric flask and the pipette for measuring out the sample are cleaned before each analysis with petroleum ether. The pipette is afterwards dried in a current of air. The petroleum ether left over in the volumetric flask after pipetting off the sample may be used over again after distillation.

Precipitation of Cholesterol Digitonide—To the petroleum ether sample in the combustion tube is added 0.2 cc. of a 1 per cent digitonin solution (in 50 per cent redistilled alcohol). The contents of the tube are allowed to evaporate at not over 60°. The best precipitate is obtained if the evaporation is made very slowly (3 to 4 hours or overnight). Under these conditions the precipitation of the cholesterol is complete.[1] If the evaporation is performed rapidly, the petroleum ether may creep up towards the neck of the tube and escape contact with the alcoholic digitonin solution at the bottom of the tube. Suction should not be employed during the evaporation as particles of dust are likely to be drawn into the tube and would not be removed by the subsequent washings. The temperature during the evaporation must not exceed 60°, as higher temperature might render the phosphatides partially insoluble and make the washing of the cholesterol digitonide precipitate incomplete.

Washing of Cholesterol Digitonide Precipitate—5 cc. of redistilled ether are added to the residue in the combustion tube and drawn up through an immersion filter stick, the lower portion of which, containing the alundum filter disk, is detachable (see Fig. 3). Several tubes can be washed, each with a separate filter stick, at the same time. The suction must be so regulated that the flow up into the filter stick is not rapid or bubbling. A total of five successive washings with 5 cc. of ether each is made, the filter stick being removed from the tube before each new addition. When the ether washing of a series of tubes is completed, the waste bottle in the vacuum line should be emptied. If this precaution is neglected, the ether vapors in the waste bottle may expand when the later hot water washings enter the bottle, and material on the end of the filter stick may be blown off by the back pressure. If this occurs at a moment when the stick is held outside the tube, the analysis will be lost.

After the last ether washing the tubes are heated on the steam bath to allow escape of any remaining ether before the washings with hot water. If the hot water is added to the tube in the presence of ether, some material may be lost by expulsion with ether vapor. When completely dry, the precipitate is washed four times with 5 cc. portions of boiling distilled water, the water being drawn through the filter stick into the empty waste bottle in the vacuum line. After the washings are completed, the lower part of the filter stick is pushed off into the bottom of the combustion tube by means of a glass rod. The water in the tube is completely evaporated by heating the tube in a steam bath, and the sample is ready for combustion.[2] The washing of a sample with ether and water requires about 5 minutes.[3]

---

[1] The Liebermann-Burchard reaction on chloroform extract of the content of the tube after this has been evaporated to dryness was found negative. Also quantitative results were uniformly obtained by analysis of cholesterol standard solution.

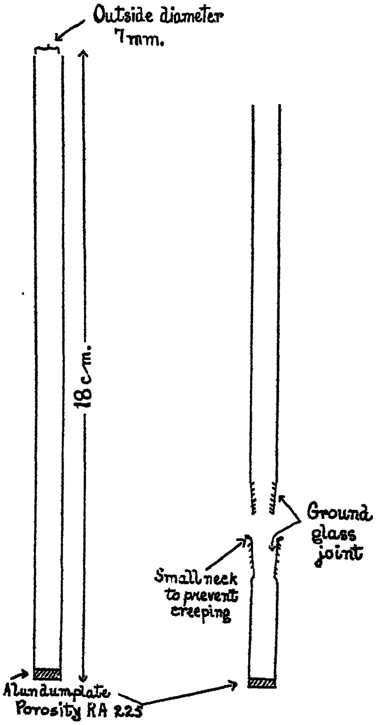


FIG. 2 FIG. 3

FIG. 2. Filter stick with porous tip not detachable.
FIG. 3. Filter stick with porous tip detachable.

---

[2] If in analysis of a sample with unusually high cholesterol content the carbon dioxide developed exceeds the amount which can be measured in the

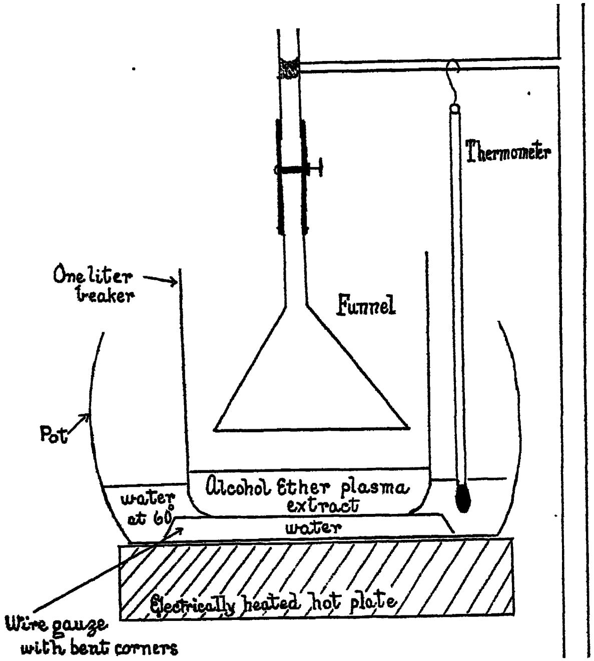

Thermometer
One liter beaker
Funnel
Pot
water at 60°
Alcohol Ether plasma extract
water
Electrically heated hot plate
Wire gauze with bent corners

FIG. 4. Arrangement for evaporating alcohol and ether from the alcohol-ether extract.

---

ordinary Van Slyke-Neill chamber, the duplicate cholesterol digitonide sample may be saved by redissolving the precipitate in hot absolute alcohol and performing the non-volatile carbon determination on an aliquot of the solution.

[3] If instead of a detachable filter stick a non-detachable one is used (see Fig. 2), the precipitate adhering to the end of the filter and to the outside of the filter stick may be redissolved and washed into the combustion tube by means of hot absolute alcohol, 1 to 2 cc. portions of which are alternately poured into the tube of the filter stick and over the outside of the tip of the filter. The absolute alcohol is afterwards evaporated on the steam bath and the residue determined by combustion. Care should be taken not to touch the stick with the fingers within 2 inches from the tip, and, in pouring the alcohol into the filter stick, to avoid drops running down the outside of the stick and getting into contact with the fingers. The use of a filter stick with a non-detachable tip is, however, quite tedious, about 20 minutes being required for the preparation of each sample. Identical results are obtained by both procedures.

Note on Combustion of Cholesterol Digitonide, Prepared As Described Above—When the 1 cc. of combustion fluid is added to the tube containing the end of the detachable filter stick, care should be taken not to deliver the fluid into the open end of the filter tube, as too little fluid will remain outside the filter to insure contact with the cholesterol digitonide precipitate left on the walls of the combustion tube. To prevent breaking of the glass filter the tube is not shaken during the combustion, but complete contact between the combustion fluid and the walls of the combustion tube is secured by vigorous lowering of the mercury leveling bulb.

If this precaution is observed, one filter stick may serve for many analyses. It is advisable to test each filter on a standard cholesterol solution before use. The alundum disks in the filter sticks, when not in use, have been kept in chromic acid mixture. Probably owing to this unnecessary precaution, crumbling of the alundum has occurred after prolonged use. New alundum disks can easily be fused into the same filter sticks.

Petroleum Ether Extraction of Residue from Alcohol-Ether Extract

For Determination of Total Fat, Free Cholesterol, Total Lipid Nitrogen, Lipid Amino Nitrogen, and Lipoid Phosphorus

Evaporation of Alcohol-Ether Extract—75 cc. of A-E Extract A are evaporated to dryness in a 1 liter beaker, the bottom of which is immersed in water at 60° (Fig. 4). The temperature limit of 60° was found to be of the greatest importance, as the reextraction of the lipids with petroleum ether was found to be incomplete if this temperature was exceeded. When the alcohol-ether solvent was evaporated at 60° or less, the reextraction of the lipids yielded very constant results, much higher than those obtained if the beaker had been heated to 75°.

It is safe never to allow the temperature to rise above 60°. To accelerate evaporation a current of air drawn through a funnel may be used with advantage (Fig. 4). About 45 minutes are required to evaporate 75 cc. of alcohol-ether to dryness under these conditions.

Reextraction of Alcohol-Ether Residue with Petroleum Ether—The lipids of the residue are redissolved by repeated extractions with petroleum ether. The first extractions are made with cold, the last ones with boiling petroleum ether. Each petroleum ether portion is filtered through a sintered glass filter (Jena, No. 17-G-4, diameter 7 cm., capacity of cup 175 cc.), ground to fit the neck of a 100 cc. calibrated volumetric flask (see Fig. 5). The filter should be of such a porosity as to allow the filtration of the residue from 75 cc. of alcohol-ether without clogging. It is usually not necessary to use suction during the filtration. If occasionally the filtration is slow, only slight suction should be used. After the extraction with petroleum ether is complete, the filter is washed repeatedly with petroleum ether, and, after cooling, the contents of the 100 cc. flask are made up to volume. Each cc. of solution represents 0.0225 cc. of plasma. The solution will be termed Petrol Extract A.

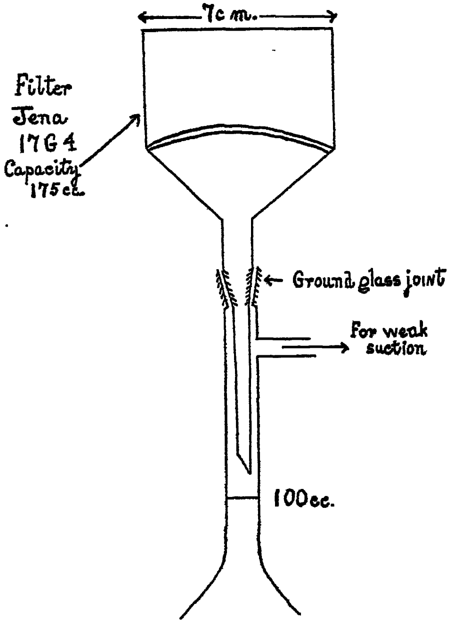


FIG. 5. Arrangement for filtering extract through porous glass funnel.

Petroleum ether has over ether an advantage in the ease with which the former is kept anhydrous. It was also found to have the following essential properties for reextraction of the plasma lipids. (1) Exclusive solution of lipoid phosphorus. No measurable amount of inorganic phosphorus, sodium glycerophosphate, or hexosediphosphate was dissolved by petroleum ether under the conditions of the extraction. As shown later, all the phosphorus extracted under these conditions from plasma, red blood cells, and tissues was found to be precipitable by acetone and magnesium chloride under the conditions used to precipitate phosphatides. (2) Entire lack of solvent power for amino acids. Petroleum ether was found to dissolve no amino nitrogen from a mixture of monoamino acids from hydrolyzed casein. (3) The cholesterol digitonide was found to be more suitable for filtering and washing when it was precipitated from petroleum ether solution than when it was precipitated from ether or alcohol solution. (4) The extraction of the lipids with petroleum ether was complete. Further extraction of the residue with ether increased the redissolved carbon by only approximately 1 per cent. The Liebermann-Burchard reaction was negative in this final ether extract.

Note on Cleaning of Glass Filter—After each filtration the glass filter is cleaned with sulfuric-chromic acid cleaning mixture, and afterwards rinsed thoroughly with water. It must be completely dried before use. The drying is accomplished by filtering successive portions of alcohol, ether, and petroleum ether through the filter.

Total Lipids

In Petrol Extract A—If the fat content of the blood is normal, two 2 cc. samples of Petrol Extract A are pipetted into combustion tubes. Each sample represents 0.045 cc. of plasma. If the plasma is lipemic, however, only a 1 cc. sample, representing 0.0225 cc. of plasma, is used. Occasionally heavy lipemia may necessitate use of still smaller samples. A portion of Petrol Extract A is then diluted with petroleum ether and a suitable aliquot is taken.

After the petroleum ether has been driven off from each tube, 2 drops of distilled water are added and evaporated to dryness. The vapors are finally swept out of the tube with a momentary current of air (4 to 5 seconds) and the residues are submitted to combustion.

In Petrol Extract B—Petrol Extract B is prepared from A-E Extract B. 20 cc. of the latter are evaporated to dryness in a 100 cc. beaker at not more than 60°. The residue is stirred up with 5 cc. portions of petroleum ether, which are passed through a sintered glass filter into a 25 cc. flask and made up to the mark. For the combustion 5 cc. portions, representing 0.032 cc. of plasma, are taken if the plasma is normal. If it is lipemic, samples of 3 or 2 cc., representing 0.0192 or 0.0128 cc. of plasma, are taken.

Free Cholesterol

For analyses of normal plasma two samples of 5 cc. each of Petrol Extract A, representing 0.1125 cc. of plasma, are pipetted into combustion tubes. The cholesterol is precipitated with digitonin and the precipitate is washed and subjected to combustion as described for the determination of total cholesterol.

For lipemic bloods, samples of 3 or 2 cc. of Petrol Extract A, representing 0.0675 or 0.045 cc. of plasma, may be taken.

Total Lipid Nitrogen

For gasometric micro-Kjeldahl analyses duplicate 10 cc. samples of Petrol Extract A are measured into 200 × 25 mm. Pyrex test-tubes. Two glass beads are added to each, and the samples are evaporated to dryness on the steam bath. The evaporation is performed slowly to prevent the petroleum ether from creeping. When the residue is dry, 1 gm. of potassium persulfate is added in substance, followed by 1 cc. of water and 1 cc. of the sulfuric-phosphoric acid-selenium digestion mixture. In adding the water and the digestion fluid, care is taken to wash down any persulfate crystals which may stick to the walls of the tube. Otherwise, error due to development of oxygen gas from undigested persulfate during the reaction with hypobromite may occur. Only gentle heating should be applied to the tube during the 1st minute of the digestion. Too intense initial heating may cause excessive foaming and deposition of material high on the inside of the tube, where contact with the digestion fluid is difficult to obtain. Even when the heating is begun carefully, it is frequently necessary to remove the tube temporarily from the flame, and, by holding the tube in an oblique position, insure contact between the digestion fluid and particles of undigested material on the walls of the tube. The digestion of the lipids was found to be complete in 20 minutes. If the selenium catalyst was omitted from the digestion mixture, digestion was slower, and nitrogen values 2 to 3 per cent too low were likely to be obtained.

Neutralization of the acid-digest is performed as described by Van Slyke (1926–27) (also Peters and Van Slyke (1932) pp. 353–358). The neutralization should be made immediately after cooling, and the tubes then closed until ready for the gasometric analysis, to prevent absorption of any ammonia from the air. For the gasometric analysis the new hypobromite reagent of Van Slyke and Kugel (1933) is used.

A blank is run with each set of reagents.

Lipid Amino Nitrogen ("Cephalin")

Emulsification of Lipids and Transfer to Manometric Chamber—Two samples of 20 cc. each of Petrol Extract A, representing 0.45 cc. of plasma, are pipetted into 150 cc. beakers and evaporated to dryness without heating the outside of the beakers above 60°.

The residue at the bottom of each beaker is brought into emulsion by addition of 2 cc. of distilled, ammonia-free (tested with Nessler's reagent) water and by stirring with a rubber-tipped glass rod. The 2 cc. of lipid suspension are poured from the beaker into the cup of the Van Slyke apparatus and slowly admitted to the chamber, allowing complete drainage of the walls of the cup. The beaker, glass rod, and cup are washed with three successive 1 cc. portions of water, making a total volume of 5 cc. Care is taken to bring into emulsion any material that might have crept up the sides of the beaker. In each of the washings the inside of the cup is scrubbed with the rubber-tipped glass rod. Even if the suspension is not homogeneous, which is sometimes the case in samples of lipemic blood, it will become so after shaking in the chamber with acetic acid. The quantitative transfer of the emulsion is easy if the material has not been heated above 60°.

Removal of Air from Mixed Solution of Lipids and Acetic Acid—1 cc. of glacial acetic acid is added exactly from an Ostwald pipette. The manometric chamber is evacuated and shaken for 1½ minutes and the extracted air is ejected. The extraction is once repeated, and an additional minute bubble of air is ejected. As the lipid suspension shows no tendency to foam, addition of caprylic alcohol has not been found necessary.

Decomposition of Amino Groups—The total reaction time necessary for complete decomposition of the cephalin amino groups was found to be 3 times that for the α-amino groups of amino acids at the same temperature (Table I and Fig. 6).

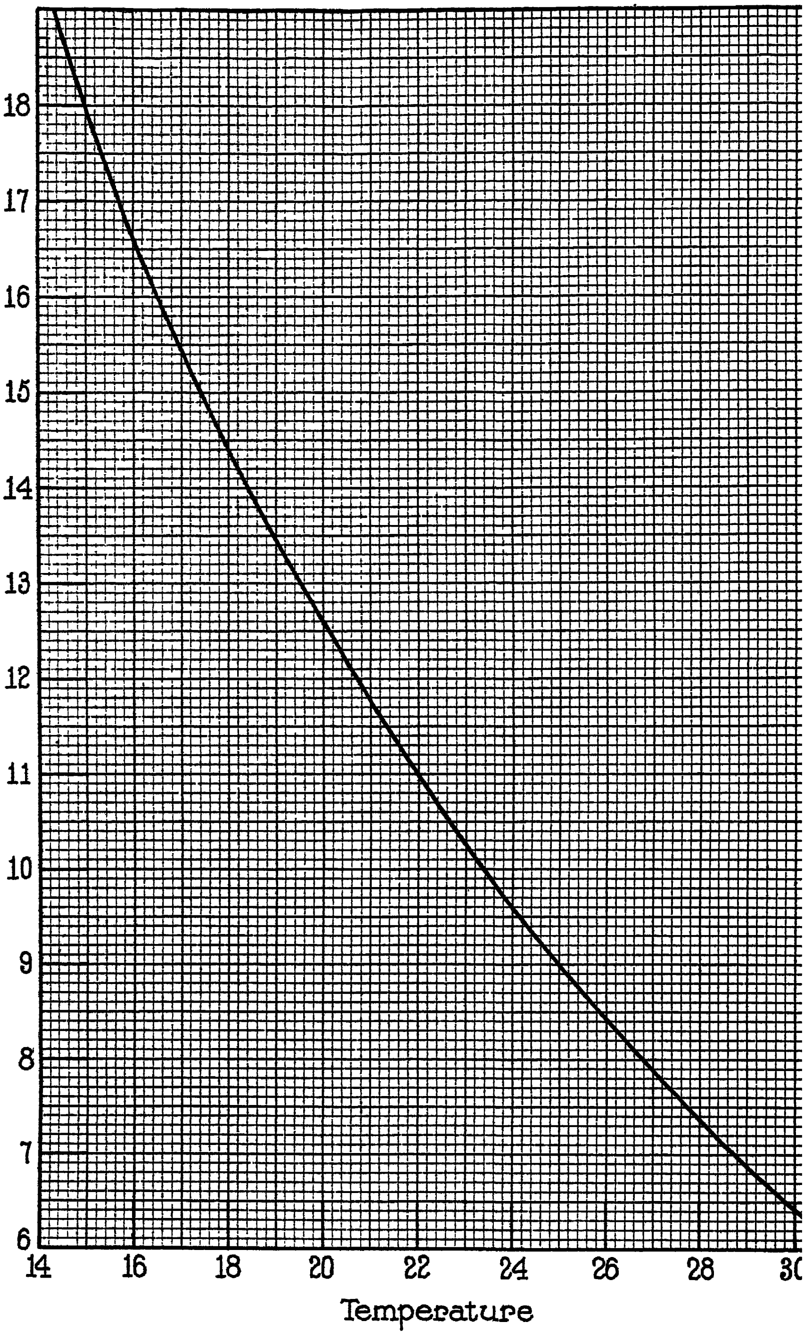


FIG. 6. Time required for reaction of cephalin amino group with nitrous acid.

2 cc. of sodium nitrite solution are measured into the chamber from an Ostwald pipette. The chamber is not evacuated immediately as in the ordinary amino nitrogen analysis but the solution is allowed to remain in the upper portion of the chamber during the first 2 minutes of the reaction time. This precaution is taken because it was found that otherwise part of the lipid solution would stick to the upper portion of the chamber and escape contact with the nitrite solution. After 2 minutes the chamber is evacuated till the mercury in it has fallen to 1 or 2 cm. above the 50 cc. mark, and is shaken for 15 seconds to insure complete mixing of the solutions. As in the ordinary amino nitrogen analysis, the reaction mixture is permitted to stand in this position until within 1 minute of the end of the reaction time (3 times that of ordinary α-amino groups (see Fig. 6)). Two or three times during the reaction period the mixture is shaken for a few seconds to insure contact with the film of solution adhering to the walls of the middle portion of the chamber. During the last minute the mixture is shaken as usual to complete the evolution of the N_2 formed. The remaining portion of the analysis is identical with that for the determination of ordinary α-amino groups.

TABLE I

Reaction Time for Amino Groups in Cephalin Suspension by Manometric Amino Nitrogen Determination of Van Slyke

Cephalin sample equivalent to 0.4380 mg. of amino nitrogen.

Period No.*	Amino nitrogen developed	Per cent of total
	mg.	
1	0.3769	86.0
2	0.4167	95.4
3	0.4340	99.1

* The temperature during the reaction was 23.6°. Each period lasted 3 minutes and 18 seconds. The gas developed in each period was collected separately.

From 25 to 30 minutes are required to emulsify the lipid residue, transfer it to the manometric chamber, and determine the amino nitrogen.

Blank—With each series of analyses the usual blank is run with 5 cc. of water instead of the lipid emulsion. Each preparation of petroleum ether used must also be tested by treating 20 cc. as described above. It is of the greatest importance to make sure that the petroleum ether is sufficiently purified to give a blank nearly as low as that of distilled water. It was found that the residue of 20 cc. of Kahlbaum's petroleum ether "for fat determination" gave a blank of approximately 120 mm. pressure at the 0.5 cc. mark. This blank was not caused by any nitrogenous substances in the petroleum ether, but was reduced to approximately the blank of distilled water when the petroleum ether had been purified as described under "Reagents."

Cleaning of Chamber after Analysis—After each series of analyses the manometric chamber is cleaned by filling it with sulfuric-chromic acid cleaning mixture, with precautions for preventing contact with the rubber connection at the bottom of the chamber, as described by Van Slyke and Neill (1924).

Lipoid Phosphorus

Principle—The lipids are digested by Neumann's wet ashing procedure, and the phosphoric acid in the digest is determined by the gasometric method of Kirk (1934).

Evaporation—Two samples of 10 cc. each of Petrol Extract A are measured into 200 × 15 mm. Pyrex digestion tubes, which have previously been calibrated at 25 cc. Each tube is constricted to 10 to 12 mm. diameter at the calibration mark and is identical with the tubes used for digestion in the manometric total base determination of Van Slyke, Hiller, and Berthelsen (1927) (see Peters and Van Slyke, 1932, p. 403). One Pyrex glass bead is added and the petroleum ether is evaporated to dryness on the steam bath. (The ordinary type of digestion tubes used for digestion of the lipids for nitrogen determination may be used instead of the calibrated tubes. In this case tubes used for phosphorus determination should be kept carefully separated from those used in the nitrogen determination, to avoid contamination with phosphoric acid from the sulfuric-phosphoric acid digestion mixture used in the Kjeldahl analyses.)

Digestion of organic matter and neutralization of the digest is carried out as described in the accompanying paper by Kirk (1934). 1 cc. of water is added to the dry residue, followed by 1 cc. of the sulfuric-nitric acid digestion mixture. Further addition of nitric acid to complete the digestion is usually unnecessary. 6 cc. of the neutralized digest, representing 0.054 cc. of plasma, are used for each analysis.

A blank must be run on the reagents, with 10 cc. of petroleum ether instead of the sample.

Calculation—The factors for calculation of the various lipid fractions are given in Table II. The factors for total phosphatides were obtained by multiplication of the phosphorus factors by 23.54.

Mg. carbon in sample = $(p_1 - p_2 - c) \times f_{\text{carbon}}$
" cholesterol in sample = $(p_1 - p_2 - c) \times f_{\text{cholesterol}}$
" lipid nitrogen in sample = $(p_1 - p_0 - c) \times f_{N_2}$
" amino nitrogen in sample = $(p_1 - p_0 - c) \times f_{\text{amino nitrogen}}$
" phosphorus in sample = $(p_1 - p_2 - c) \times f_{\text{phosphorus}}$
" phosphatide " " = $(p_1 - p_2 - c) \times f_{\text{phosphatide}}$
" substance per 100 cc. plasma = (mg. substance in sample) × (100/cc. plasma represented in sample)

The c correction in each analysis represents the blank on the whole procedure.

The f factors are given in the original papers on manometric carbon, nitrogen, phosphorus, and NH_2 determination, but for convenience those which apply to the determinations described in this paper are collected in Table II.

For convenience in calculation of the mg. per cent values of the different lipid fractions in normal and lipemic plasma Table III is included, giving the volume of plasma represented in each sample.

Calculation of Total Lipid—An *approximate* value for the total lipids can be obtained by multiplying the total petroleum ether-soluble carbon by 1.3, on the assumption that the carbon content of the mixture is 77 per cent.

However, the lipids represented include those with carbon contents varying from 83.9 per cent in the case of cholesterol to 66.2 per cent in the case of cephalin. Hence it is obvious that the total lipid value cannot be calculated accurately by using a single calculation factor. In the publication of the manometric carbon method factors for calculation of "mixed plasma lipids" were given in Table I of that paper. It was, however, pointed out that these factors could be used only if the sample analyzed consisted of a mixture of cholesterol with either the neutral fats or with the fatty

TABLE II

Factors for Calculation of Carbon, Lipids, and Phosphorus from P_{CO_2} Obtained at 2 Cc. Volume after Wet Combustion, and of Total Lipid Nitrogen and Lipid Amino Nitrogen from P_{N_2} Obtained at 0.5 Cc. and 2 Cc. Volume

Multiplication of P_{CO_2} or P_{N_2} by the proper factor gives the mg. of substance in the sample.

Temperature	Factors by which P_{CO_2} is multiplied to give						Factors by which P_{N_2} is multiplied to give			
	Carbon	Cholesterol when the digitonide is oxidized	Fatty acids	Neutral fat	Lipoid phosphorus	Total phosphatide	Total lipid nitrogen by micro-Kjeldahl		NH_2-nitrogen by nitrous acid reaction	
	$a = 2.0$	$a = 2.0$	$a = 2.0$	$a = 2.0$	$a = 2.0$	$a = 2.0$	$a = 0.5$	$a = 2.0$	$a = 0.5$	$a = 2.0$
°C.										
15	0.001474	0.000579	0.001941	0.001921	0.00005190	0.001222	0.000813	0.003250	0.000390	0.001561
16	66	576	31	11	62	15	810	41	389	55
17	58	572	21	01	34	09	807	27	387	49
18	50	569	11	0.001890	06	02	804	15	386	44
19	42	566	00	80	0.00005078	0.001195	800	01	385	38
20	35	563	0.001891	71	53	90	0.000798	0.003192	383	33
21	27	560	81	60	25	83	795	80	382	27
22	20	557	71	51	00	77	792	68	380	22
23	12	554	61	41	0.00004972	70	789	56	379	16
24	05	552	52	32	47	64	786	45	378	11
25	0.001398	549	42	23	23	58	784	36	376	06
26	91	546	33	14	0.00004899	52	781	24	375	00
27	85	544	25	06	77	47	779	16	374	0.001495
28	78	541	16	0.001797	52	42	776	04	372	90
29	72	539	08	89	31	36	773	0.003092	371	85
30	65	536	0.001799	80	06	31	770	80	370	80
31	59	534	91	72	0.00004785	26	768	71	368	74
32	53	531	84	65	64	21	765	60	367	69
33	48	529	76	57	46	17	762	50	366	64
34	0.001342	0.000527	0.001769	0.001750	0.00004725	0.001112	0.000760	0.003040	0.000365	0.001459

acids obtained after saponification, as in Stoddard's method, since the factors for neutral fat and fatty acids differ by only 1 per cent. At the same time the assumption must be made (as does Bloor) that the mixture consist of 2 parts of fat to 1 part of cholesterol.

TABLE III

Volume of A-E Extract A and Petrol Extract A Used for Individual Lipid Analyses of Normal and Lipemic Plasma

Normal	Lipemic
Total cholesterol	
For saponification 5 cc. A-E.* After saponification 10 cc. aliquots of petroleum ether solution analyzed ~ 0.06 cc. plasma	For saponification 5 cc. A-E. After saponification 5 cc. aliquots of petroleum ether solution analyzed ~ 0.03 cc. plasma
Free cholesterol	
5 cc. P. E.† ~ 0.1125 cc. plasma	3 cc. P. E. ~ 0.0675 cc. plasma
Total fat	
2 cc. P. E. ~ 0.045 cc. plasma	1 cc. P. E. ~ 0.025 cc. plasma
Lipid nitrogen	
10 cc. P. E. ~ 0.225 cc. plasma	Same
Lipoid phosphorus	
10 cc. P. E. used for digestion. 6 cc. sample of diluted digest analyzed ~ 0.054 cc. plasma	Same
Lipid amino nitrogen	
20 cc. P. E. ~ 0.45 cc. plasma	Same

* A-E represents A-E Extract A.

† P. E. represents Petrol Extract A.

We have found it convenient in the daily analyses to report the total lipid values simply as "total lipid carbon." If instead of the total lipid carbon value the accurate total lipid value is desired, this can be calculated by summation of the individual lipid fractions determined in the separate analyses. The fraction remaining after the carbon values for cholesterol, cholesterol esters, and phosphatides have been subtracted from the "total lipid carbon" can be assumed to consist chiefly of neutral fats with a carbon content of 76.7 per cent. The calculation will be illustrated by an example.

Total lipid carbon	Cholesterol		Lipoid phosphorus
	Esters	Free	
mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
1459	524	194	19.85

19.85 mg. lipoid phosphorus corresponds to 19.85 × 23.54 = 468 mg. phosphatide or (468 × 66.2)/100 = 310 mg. phosphatide carbon
524 mg. cholesterol esters contain (524 × 82.5)/100 = 432 mg. carbon
194 " " contain (194 × 83.9)/100 = 163 mg. carbon
Total lipid carbon from phosphatides, cholesterol esters, and cholesterol = 905 mg. carbon
Carbon from neutral fat = 1459 − 905 = 554 mg.
554 mg. carbon corresponds to (554 × 100)/76.7 = 723 mg. neutral fat
Total lipid, 468 (phosphatides) + 524 (cholesterol esters) + 194 (free cholesterol) + 723 (neutral fat) = *1909 mg. per cent*
Total lipid calculated by approximate factor = 1.3 × total carbon = 1.3 × 1459 = *1897 mg. per cent*

EXPERIMENTAL

The results of saponification of a standard solution of cholesterol palmitate are given in Table IV. In Table V values obtained by extraction of the same plasma by different methods are presented. The reflux extraction procedure with the alcohol-ether mixture of Man and Gildea as described in this paper gave the highest yield. In Table VI the results of duplicate extractions of the same plasma with this procedure are given. The quantitative recovery of free cholesterol, cholesterol esters, lipid nitrogen, lipid amino nitrogen, and lipoid phosphorus is presented in Table VII. The lipids were dissolved in alcohol-ether and added to the extraction fluid before the reflux extraction was started. The cholesterol, cholesterol palmitate (prepared according to Page and Rudy (1930)), and a phosphatide preparation from egg yolk, freshly prepared according to MacLean and MacLean (1927), were added to separate aliquots of the same plasma.

TABLE IV

Saponification of Cholesterol Palmitate

Cholesterol in sample	Cholesterol found	Deviation from theoretical
mg.	*mg.*	*per cent*
0.2490	0.2542	+2.0
0.2490	0.2528	+1.5
0.2490	0.2440	−2.0
0.2490	0.2546	+2.2
0.2490	0.2528	+1.5

TABLE V

Comparison of Results Obtained by Extraction of Same Plasma by Different Methods

Results are expressed in mg. per 100 cc. of plasma.

	Plasma A				Plasma B			
	Gasometric			Colorimetric	Gasometric			Colorimetric
	Total fat	Free cholesterol	Total cholesterol	Total cholesterol	Total fat	Free cholesterol	Total cholesterol	Total cholesterol
Bloor's procedure; extraction with equal volumes of absolute alcohol and chloroform	603	27.5	*	230.7	763	42.1	*	304.6
Bloor's procedure; extraction with alcohol-ether (3:1)	597	30.8	199.5	235.5	759	42.9	233	286.9
Mann and Gildea's reflux extraction with alcohol-ether (3:1), as described in text	600	31.4	202.0	251.1	835	35.1	252	308.3

* Values for total cholesterol were not obtainable in the chloroform-alcohol extract, probably owing to splitting of the chloroform during the saponification.

TABLE VI

Comparison of Results Obtained by Duplicate Extractions of Same Plasma by Mann and Gildea's Reflux Procedure

Values represent mg. per 100 cc. of plasma.

Material	Total fat	Total cholesterol	Free cholesterol	Lipid NH_2-nitrogen	Total lipid nitrogen	Lipoid phosphorus	Phosphatide calculated from phosphorus
Plasma A	2061	582	440	6.83	18.06	11.65	274
	2072	595	445	6.44	17.44	11.51	271
Plasma B	1360						
	1342						
	1305						
	1358						

TABLE VII

Recovery by Gasometric Procedure of Cholesterol, Cholesterol Esters, Cephalin Nitrogen, Lipid Nitrogen, and Lipoid Phosphorus Added to Plasma

Values represent mg. per 100 cc. of plasma.

	Free cholesterol	Total cholesterol	Lipid amino nitrogen*	Lipid nitrogen*	Lipoid phosphorus*
Original	67.5	162.6	2.64	12.70	3.51
Added	238.3	105.4†	3.26	10.97	25.40
Calculated	305.8	268.2	5.90	23.67	28.91
Found	296.9	274.1	6.18	22.84	28.47
Deviation, *mg. per cent*	−8.9	+6.9	+0.28	−0.83	−0.44
" *per cent*	−2.91	+2.20	+4.74	−3.50	−1.52

* The lipid amino nitrogen, total lipid nitrogen, and lipoid phosphorus were added in the form of phosphatide prepared from egg according to MacLean and MacLean (1927).

† The cholesterol ester preparation used for addition was cholesterol palmitate, prepared according to Page and Rudy (1930).

Average Percentage Errors

The average percentage errors in duplicate analyses of the same plasma extract are the following: total lipid, 0.50; free cholesterol, 0.50; total cholesterol, 2 (error chiefly incurred in saponification); total lipid nitrogen, 0.75; lipid amino nitrogen ("cephalin nitrogen"), 3; and lipoid phosphorus, 0.75.

Comparison of Gasometric Phosphorus Determinations in Petroleum Ether Extract with Determinations of Phosphatides Precipitated by Acetone-$MgCl_2$ According to Procedure of Bloor (1929)

As mentioned in the introduction to this paper it was not found possible to obtain quantitative results by applying the acetone-$MgCl_2$ precipitation of Bloor to the small amounts of phosphatide ordinarily used for the gasometric carbon method. Only about 85 per cent of the phosphatide carbon was recovered after precipitation of samples containing about 0.5 mg. of phosphatide. Our results in this respect agree with the observations of Boyd. When larger samples were used for precipitation, however, as described by Bloor, the precipitation of the phosphatide was found to be practically complete, with standard solutions of pure phosphatides, with mixtures of pure phosphatides and other lipids, and with extracts of plasma, red blood corpuscles, and tissues. We were thus able fully to confirm Bloor's statement that, under the conditions prescribed by him, only a small fraction of the phosphatide (in our analyses approximately 2.2 per cent) escapes precipitation. In preparation of the samples for precipitation the precaution of avoiding heating of the alcohol-ether extract residue above 60° was strictly observed. Also filtration of the petroleum ether extract through a sintered glass filter was preferred to separation of impurities by centrifugation and decantation.

It was soon found, however, in analyses of plasma extracts, that if the acetone-$MgCl_2$ precipitate was redissolved in moist ether, according to Bloor's directions, the amount of lipid in the final ether solution was definitely less than the amount of phosphatide calculated from the phosphorus content of the original petroleum ether extract (see Table VIII). The precipitation of the phosphatides by acetone-$MgCl_2$ was practically complete, but *part of the precipitated phosphatide did not redissolve in moist ether*, in spite of the fact that liberal time and care were used in the attempt to bring all the precipitate into solution.

The question arose whether this insoluble portion represented a definite lipid fraction. The phosphatides in the petroleum ether extract from a large volume of human blood were therefore precipitated with acetone-$MgCl_2$ according to Bloor. As much as possible of the precipitate was redissolved in moist ether. A residue remained and was dissolved in hot alcohol. Aliquots of the alcoholic solution were analyzed for nitrogen and phosphorus. The N:P ratio found was 0.875 (instead of the usual ratio for cephalin and lecithin of 0.438), showing this fraction to be a diaminomonophosphatide.

TABLE VIII

Comparison of Phosphatide Values of Plasma Obtained by Bloor's Isolation Method and Oxidative Procedure with Values Calculated from Gasometric Phosphorus Estimations Performed on Same Petroleum Ether Extracts

Hospital No.	Plasma phosphatide found		Deviation	
	Acetone-$MgCl_2$ precipitate, redissolved in moist ether. Determined by Bloor's oxidation	Total precipitate calculated as gasometric phosphorus × (100/4.17)		
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
8634	350	476	116	24.4
7049	484	549	65	11.8
8740	442	530	88	16.6
8520	137	197	60	30.4

TABLE IX

Analyses of Egg Phosphatide Solution, Concentrated to Dryness below 60° and Redissolved in Petroleum Ether

Total phosphatide carbon in sample	Phosphatide carbon not pptd. by acetone-$MgCl_2$		Phosphatide carbon precipitated by acetone-$MgCl_2$			Phosphatide carbon accounted for	
	*a**		Total ppt.	Soluble in moist ether *b*	Residue insoluble in moist ether *c*	*a* + *b* + *c*	
mg.	*mg.*	*per cent of total*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1.331	0.024	1.80	1.244	1.180	0.060	1.264	94.9
1.331	0.027	2.03	1.234	1.150	0.076	1.253	94.2
1.331†	0.018	1.35	1.236	1.203	0.033	1.254	94.2

* Calculated from gasometric phosphorus analysis (P × (66.2/4.17)). If the supernatant fluid contained non-phosphatide impurities, the carbon would be higher than calculated from the phosphorus. Possibly error from this source causes the 5 per cent deficit in total carbon accounted for (last column).

† To this sample were added 2.1 mg. of cholesterol and 4.0 mg. of palmitic acid.

In Table IX the results of precipitation of a prepared solution of phosphatides with acetone-$MgCl_2$ are given. The phosphatide preparation was prepared from egg yolk according to MacLean and contained both cephalin and lecithin. The analyses were performed by the gasometric carbon method.

In Table X the results of analyses of extracts of plasma, red blood corpuscles, and liver tissue by acetone-$MgCl_2$ precipitation and by gasometric phosphorus determination are given. The petroleum ether extracts were prepared as described previously in this paper.

TABLE X

Analyses of Blood and Tissue Extracts Obtained by Refluxing with Alcohol-Ether, Concentrating Filtrate to Dryness below 60°, and Extracting Residue with Petroleum Ether

Material	Amount of material represented in extract sample	Phosphatide pptd. by acetone-$MgCl_2$					Total phosphorus in petroleum ether extract per 100 cc. or gm. material	Ratio, mg. phosphatide† to mg. P
		Part soluble in moist ether	Residue insoluble in moist ether	Total				
				Carbon calculated as $a + b$	Carbon per 100 cc or gm material	Lipid per 100 cc or gm. material*		
		a	b					
		mg. C.	*mg. C.*	*mg. C.*	*mg. C*	*mg. lipid*	*mg. P*	
Human plasma.......	1.125 cc.	0.998	0.184	1.182	105	159	6.91	23.00
" red blood cells...............	0.450 "	0.663	0.051	0.714	159	240	10.00	23.98
Dog liver...........	1.105 gm.	2.011	0.351	2.362	2240	3382	152.3	22.2

* Lipid calculated as carbon × (100/66.2).

† "Phosphatide" = (100/66.2) × carbon in precipitate obtained with acetone and $MgCl_2$; P = total phosphorus in petroleum ether solution.

In the analyses of Table X an average of 2.2 per cent (in no case over 3 per cent) of the total petroleum ether-soluble phosphorus was found in the supernatant fluid after the acetone-$MgCl_2$ precipitation. This observation, that the petroleum ether-soluble phosphorus in extracts of plasma, red blood corpuscles, and liver tissue is precipitated to the same extent (97.8 per cent) as standard solutions of phosphatides, is evidence that all this extract phosphorus represents phosphatides, and that the petroleum ether extract prepared under the conditions outlined for the method does not contain other phosphorus compounds in measurable amounts.

If the above phosphatide to phosphorus ratios are corrected for the small phosphatide fraction left in the supernatant fluid, the ratios for plasma, red blood cells, and liver tissue become respectively 23.51, 24.41, and 22.70, and give an average ratio of 23.54.

In order to calculate phosphatides from the phosphorus of the petroleum ether extract the phosphorus values should therefore be multiplied by 23.5. The lipid figures obtained from the petroleum ether-soluble phosphorus are definitely higher than those obtained from the acetone-$MgCl_2$ precipitate redissolved in ether by the procedure of Bloor, because the total phosphorus values include the diaminophosphatide fraction which, after the precipitation, is insoluble in moist ether, and also include the small portion of phosphatide which, because of slight but measurable solubility, escapes precipitation with acetone-$MgCl_2$.

Our corrected phosphatide to phosphorus ratio of 23.5 found in plasma approximates the theoretical ratio, 23.98 for stearyllinolyl cephalin. Results to be reported later show in fact that the lipid amino nitrogen of human plasma is sufficiently high to indicate that nearly all the phosphatide may be cephalin.

Note on Application of Gasometric Carbon Method to Analyses of Phosphatides Precipitated with Acetone-$MgCl_2$ by Bloor's Procedure

Although the phosphatides are more exactly estimated from the phosphorus content of the petroleum ether extract, the fact that 98 per cent of the phosphatides can be precipitated with acetone-$MgCl_2$ makes the precipitation procedure also applicable, provided phosphatide samples of sufficient size are used, as described by Bloor, and provided also that the phosphatide fraction insoluble in moist ether is included in the combustion. Comparison of the carbon of the acetone-$MgCl_2$ precipitate with the total petroleum ether-soluble phosphorus may prove of value if knowledge of the C:P ratio in a phosphatide mixture is desired

In determining the amount of phosphatide in the acetone-$MgCl_2$ precipitate we have found it advantageous to replace Bloor's dichromate oxidation and titration method by the gasometric carbon estimation. The possibility of error from reduction of some of the dichromate by HCl liberated from $MgCl_2$ in the precipitate does not affect the gasometric carbon method. This source of error is avoided when, as in Bloor's method, only the phosphatides redissolved from the precipitate by moist ether are oxidized. The error might, however, attain importance if, in order to include the diaminophosphatide, the whole precipitate with its magnesium chloride were subjected to the Bloor oxidation.

The following procedure for estimation of the precipitated phosphatides by gasometric carbon determination was found satisfactory.

A sample of filtered Petrol Extract A, containing about 2 mg. of phosphatides, is measured into a combustion tube and concentrated to 2 cc. volume. The precipitation with acetone-$MgCl_2$ and the subsequent washing of the sample are carried out exactly as described by Bloor.

If a special manometric Van Slyke-Neill chamber calibrated at 10 cc. volume (Van Slyke, Page, and Kirk, 1933) is available, the total phosphatide precipitate can be burned as one sample. The small amount of acetone left in the combustion tube after the last washing is evaporated in the steam bath. As magnesium chloride tends to crystallize out together with some acetone, it is in this analysis especially important to observe the precaution of adding a few drops of distilled water when the contents of the tube are apparently dry, and to continue the heating in the steam bath until also the water has evaporated. The chlorine vapors which are liberated during the combustion do not interfere with the analysis. Most of the vapors are absorbed in the moisture which collects in the bulbs of the connection tube during the combustion. A combustion period of 2.5 minutes is sufficient.

If only the ordinary Van Slyke-Neill chamber, calibrated at 2 cc. volume, is available, as much of the precipitate as will dissolve in moist ether is brought into solution and the ether is transferred to a 10 cc. volumetric flask. In order to obtain an absolutely clear and homogeneous solution a few drops of absolute alcohol are added to the moist ether solution before the sample is made up to volume. 3 cc. aliquots of the moist ether solution are pipetted into combustion tubes and the solvent is evaporated. The residue in the combustion tube in which the precipitation was performed, consisting of the phosphatide fraction insoluble in moist ether, is likewise evaporated to dryness and the carbon content determined gasometrically. The total phosphatide value of the precipitate is easily calculated from the sum of the non-volatile carbon in the moist ether solution and in the magnesium chloride residue.

SUMMARY

Microgasometric methods are described for total lipids, total and esterified cholesterol, phosphatides, lipid amino nitrogen (cephalin), and total lipid nitrogen. The principles of these methods are outlined in the introduction.

For total lipid estimation alone, 0.2 cc. of plasma suffice for duplicate analyses.

For complete estimation in duplicate of the different lipid fractions 3 cc. of plasma are required.

The practice of estimating blood phosphatides in the solution obtained by precipitating them with acetone-$MgCl_2$, and redissolving in moist ether, has been found to give low results, because of the presence of a diaminophosphatide which does not redissolve in moist ether. If the entire acetone-$MgCl_2$ precipitate is determined by carbon combustion, however, the results agree with those of the total lipoid phosphorus estimation.[4]

BIBLIOGRAPHY

Bloor, W. R., *J. Biol. Chem.*, **17**, 377 (1914); **82**, 273 (1929).

Boyd, E. M., *J. Biol. Chem.*, **91**, 1 (1931).

Katsura, S., Hatakeyama, T., and Tajima, K., *Biochem. Z.*, **257**, 22 (1933).

Kirk, E., *J. Biol. Chem.*, **106**, 191 (1934).

MacLean, H., and MacLean, I. S., Lecithin and allied substances; The lipins, London, 2nd edition (1927).

Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, **99**, 43 (1932–33).

Page, I. H., and Rudy, H., *Biochem. Z.*, **220**, 304 (1930).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **2**, 386 (1932).

Shope, R. E., *J. Biol. Chem.*, **80**, 125 (1928).

Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926–27); **83**, 425 (1929).

Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, **74**, 659 (1927).

Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, **102**, 489 (1933).

Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).

Van Slyke, D. D., Page, I. H., and Kirk, E., *J. Biol. Chem.*, **102**, 635 (1933).

Windaus, A. L., *Ber. chem. Ges.*, **42**, 238 (1909).

---

[4] The special apparatus required for the methods described in this paper can be obtained from Eimer and Amend, New York.

STUDIES OF THE FAT OF HUMAN MILK

BY A. W. BOSWORTH

(From the Laboratory of Physiological Chemistry, the Ohio State University, Columbus)

(Received for publication, April 12, 1934)

Through the very generous cooperation of Dr. J. R. Thompson of St. Ann's Maternity Hospital, Cleveland, a sufficient amount of breast milk has been obtained to give 3 pounds of pure breast milk fat. This has been subjected to the same scheme of examination for unsaturated acids used in the studies of cow's milk fat as reported in a recent communication from this laboratory (1).

The presence of several hitherto unreported acids has been inferred from a study of the curves derived from the iodine numbers of the methyl ester fractions, and additional evidence has been secured to establish the presence of all but one of these acids. The presence of highly unsaturated acids of the arachidonic acid type has been demonstrated by the separation of ether-insoluble polybromides. The presence of saturated acids of greater molecular weight than that of stearic acid has been shown by the separation from acetone of saturated acids with a saponification equivalent of 339.

EXPERIMENTAL

Preparation of Fat—All of the milk was secured from mothers who were living in the hospital at the time the milk was collected and who were receiving a varied diet of fruit, vegetables, meat, fish, milk, salads, and iodine and cod liver oil when necessary. The cream, as removed from the milk, was slightly acidulated, cooled to 10°, and churned. The butter thus obtained was melted, filtered through cheese-cloth, then through filter paper, and washed with water several times, the last traces of water being removed by heating under diminished pressure. The fat thus obtained was examined and the following data secured.

Melting point, °*C*	32
Saponification No	205.1
Reichert-Meissl No	2.5
Polenské No	0.1
Iodine No	56.22
Unsaponifiable matter, *per cent*	1.13

Preparation of Methyl Esters—1 kilo of the fat was esterified by refluxing for 24 hours with 2000 cc. of methyl alcohol containing 2 to 3 per cent of dry hydrochloric acid gas. When the esterification was completed, water was added until an ester layer was obtained, and the lower layer (containing water, methyl alcohol, glycerol, and most of the methyl butyrate and methyl caproate) was then siphoned off. The esters were transferred to a separatory funnel and washed twice with distilled water.

Fractional Distillation of Esters—The esters were subjected to fractional distillation four times at a pressure of 15 mm. The first distillation gave five fractions. During the second distillation fractions were separated at 10° intervals and during the third and and fourth distillations fractions were separated at 5° intervals, except for the first three fractions which were separated at 10° intervals on account of the very small yields. The operations were conducted in the same apparatus, and with the same technique, employed in the work previously reported (1). As a result, thirty fractions were obtained.

Preliminary Examination of Fractions—The data obtained by an examination of the thirty fractions will be found in Table I. In connection with these data it should also be recorded that the esters of butyric and caproic acids, which were removed with the methyl alcohol and glycerol when the mixed esters were washed with water, were recovered and examined superficially, simply as a check upon their presence in the fat under examination.

The weights of the fractions up to and including the C_{10} fraction, together with the Reichert-Meissl and Polenské numbers of the original fat, indicate that the fatty acids of low molecular weights are present in the fat from human milk in much smaller quantities than in the fat from cow's milk. The lauric acid (C_{12}) fraction shows a distinct maximum, which is a contrast to the opposite finding for butter fat. The C_{14}, C_{16}, and C_{18} fractions merged, no maximum for the C_{14} or the C_{16} series being obtained. The last two fractions (Fractions 29 and 30), with acids of mean molecular weights greater than 284, indicate the presence of acids of the C_{20} and possibly the C_{22} series.

TABLE I

Results of Fractional Distillation of Methyl Esters of Fat from Human Milk, 1000 Gm. Fat Taken

Series	Fraction No.	B. p. at 15 mm.	Weight	Mean mol. wt. of acid	I No. of esters
		°C.	*gm.*		
C_6–C_8	1	80	0.42	137.8	1.35
C_8–C_{10}	2	80– 90	1.17	157.1	2.27
	3	90–100	1.09	157.4	2.30
	4	100–105	1.42	165.6	2.48
	5	105–110	1.00	168.1	2.40
	6	110–115	1.25	170.6	2.48
C_{10}	7	115–120	3.91	172.3	2.83
C_{10}–C_{12}	8	120–125	4.42	177.0	2.60
	9	125–130	3.50	181.0	2.17
	10	130–135	3.50	184.8	2.06
	11	135–140	3.91	190.7	2.01
C_{12}	12	140–145	22.00	195.9	0.82
	13	145–150	11.25	202.7	2.26
C_{12}–C_{14}	14	150–155	14.17	205.7	3.14
	15	155–160	8.33	209.8	6.24
	16	160–165	9.18	217.0	6.56
	17	165–170	9.92	220.2	8.01
C_{14}	18	170–175	13.50	226.6	9.85
C_{14}–C_{16}	19	175–180	22.33	235.4	12.49
	20	180–185	42.00	238.0	15.15
	21	185–190	45.83	248.2	20.40
	22	190–195	66.75	250.3	28.40
C_{16}–C_{18}	23	195–200	112.17	260.4	40.86
	24	200–205	138.33	265.4	58.46
	25	205–210	244.17	276.6	76.98
	26	210–215	145.58	277.1	85.97
	27	215–220	12.17	280.0	95.68
C_{18}	28	220–225	4.42	284.1	103.10
C_{18}–C_{20}	29	225–230	4.25	297.2	113.20
C_{20}–C_{22}	30	230–285	10.75	351.0	129.70

Distribution of Unsaturated Acids—The iodine numbers of the fractions have been plotted as Curve 1 (Fig. 1). It will be noted that an unsaturated acid or acids occur in the first fraction, in-

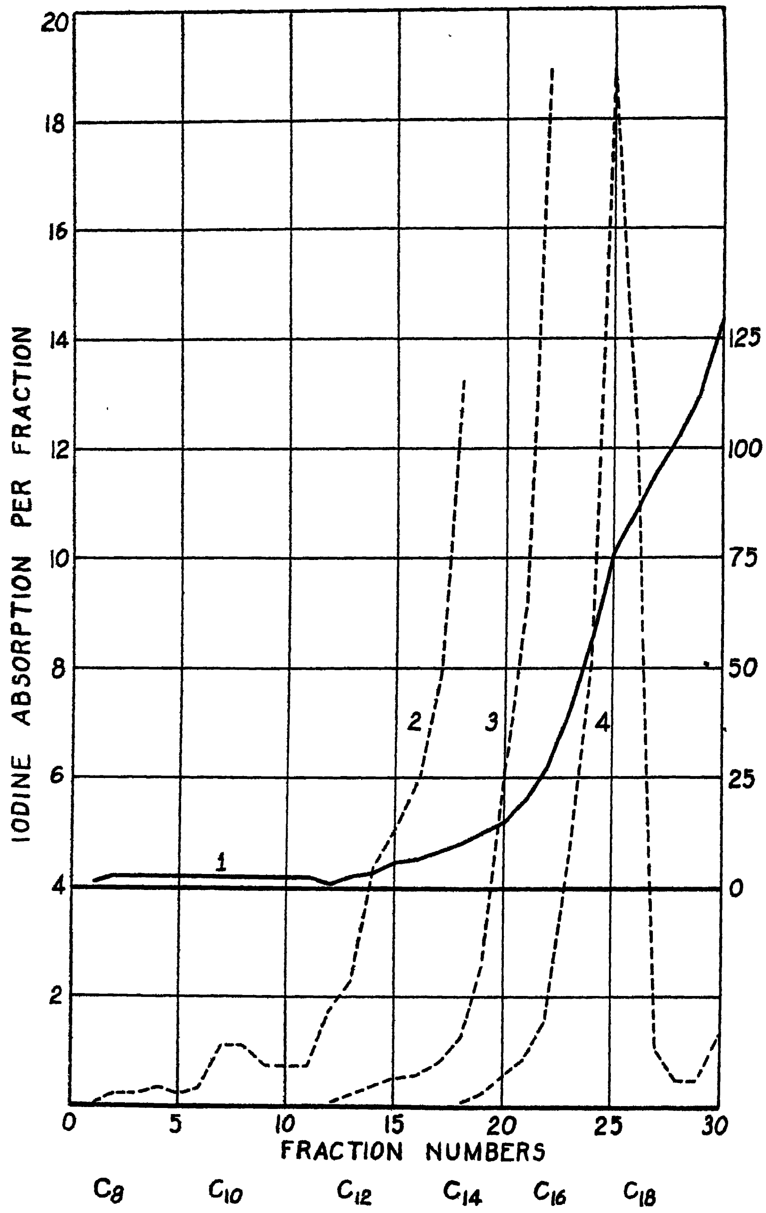


FIG. 1. Curves derived from the iodine numbers of the several fractions. Curve 1, iodine number; Curve 2, iodine absorption per fraction; Curve 3, iodine absorption per fraction /10; Curve 4, iodine absorption per fraction /100.

creasing to a maximum in the C_{10} fraction and decreasing to a minimum in the C_{12} fraction; from this point there is a gradual increase to the end. The high iodine numbers of the fractions above C_{18} indicate the presence of unsaturated acids of the C_{20} or higher series.

The total iodine absorption values for the several fractions, as calculated by the formula, iodine absorption = (weight of fraction × iodine number)/100, have been plotted as Curves 2 to 4 (Fig. 1), Curve 3 being plotted at one-tenth and Curve 4 at one-hundredth the scale used for Curve 2.

Curve 2 shows a maximum (slight) at the C_{10} point, indicating the presence of decenoic acid ($C_{10}H_{18}O_2$). The gradual upward slope of Curve 3 from the C_{12} to the C_{14} point indicates the presence of tetradecenoic acid ($C_{14}H_{26}O_2$). The abrupt change in the upward slope of Curve 3 between the C_{14} and C_{16} points, together with a second change in the slope of the curve at the C_{16} point (as shown in Curve 4), indicates the presence of hexadecenoic acid ($C_{16}H_{30}O_2$). The very high level of the maximum in Curve 4 between the C_{16} and C_{18} points indicates the presence of unsaturated acids of the C_{18} series. The fact that this maximum occurs below the theoretical boiling point for the methyl ester of oleic acid seems to be evidence of the presence of another unsaturated acid of the C_{18} series, possibly linoleic acid ($C_{18}H_{32}O_2$). The abrupt drop in the slope of the curve above C_{18} with an upward slope towards the end of the series is again evidence of the presence of unsaturated acids of the C_{20} or higher series.

Separation of Unsaturated Fatty Acids—The lead soap-ether method will not give a satisfactory separation of a C_{10} unsaturated acid, and the very small amount of material at hand prevented the isolation of this acid as the bromide of its methyl ester. When the unsaturated acid in Fractions 5 to 9 inclusive is calculated from the iodine numbers, it is found that the amount of decenoic acid in the original fat was not less than 0.024 per cent. This method of calculation seems justifiable in view of the findings for butter fat previously reported (1).

A C_{14} unsaturated acid (tetradecenoic acid) could not be separated by the lead soap-ether method in this case; but, by combining all of the remaining portions of Fractions 13 to 19 inclusive and brominating in cold ether, a bromide of a methyl ester was obtained by fractional distillation, which, upon reduction with zinc and subsequent saponification, gave an acid with a molecular weight of 224 and an iodine number of 108.4. As the theoretical figures for the molecular weight and iodine number of tetradecenoic acid are 226 and 112 respectively, it is believed the figures secured are ample proof of the presence of this acid in the fat of human milk. Assuming the unsaturated acid present in Fractions 13 to 19 to be tetradecenoic acid, it is found by calculations based upon the iodine numbers and weights of the fractions that the original fat must have contained not less than 0.576 per cent of that acid.

TABLE II

Data Obtained from Examination of Unsaturated Acids

Fraction No.	B. p. at 15 mm.	Mean mol. wt.	I No.	Method used to obtain acids
	°C.			
16–19	160–180	224	108.4	Bromination
22	190–195	257	95.0	Lead soap-ether
23	195–200	272	95.0	" " "
24	200–205	275	95.0	" " "
25	205–210	287	111.2	" " "
26	210–215	291	101.8	" " "
27	215–220	297	109.5	" " "
28	220–225	*	*	" " "
29	225–230	*	*	" " "
30	230–285	336	146.0	" " "

* Yield not sufficient for analysis.

The unsaturated acids obtained from Fraction 22 by the lead soap-ether method of separation had a molecular weight of 257 and an iodine number of 95, which are close to the theoretical figures for hexadecenoic acid ($C_{16}H_{30}O_2$); *i.e.*, a molecular weight of 254 and an iodine number of 100. An attempt was made to separate a pure hexadecenoic acid by first converting the acids, as mentioned above, to their methyl esters, then to the brominated esters, and then by fractional distillation of these brominated esters. The presence of oleic acid, and the decomposition of its brominated ester during fractional distillation, proved this method of separation to be inadequate in this case. Lack of material prevented a continued effort to separate pure hexadecenoic acid. The molecular weights and iodine numbers of the unsaturated acids separated from Fractions 23 and 24 (see Table II) might be taken as further evidence of the presence of hexadecenoic acid. The results so far obtained, therefore, lead to the belief that hexadecenoic (palmitoleic) acid may be a constituent of the fat of human milk.

The molecular weight and the iodine number for the unsaturated acid obtained from Fraction 25 indicate the presence of a C_{18} acid with a higher degree of unsaturation than that of oleic acid. This point will be discussed again in connection with the figures secured as the result of the barium soap-benzene separation.

TABLE III

Data Obtained from Examination of Unsaturated Acids As Separated from Several Fractions by Lead Soap-Ether Method, Followed by Barium Soap-Benzene Method

Fraction No.	B. p. at 15 mm.	Acids from barium soaps			
		Insoluble in benzene		Soluble in benzene	
		Mean mol. wt.	I No.	Mean mol. wt.	I No.
	°C.				
24	200–205	283	80.2	284	104.0*
25	205–210	281	96.3	282	152.4*
26	210–215	283	96.7	286	115.1*
27	215–220	289	97.4	†	†

* The tetrabromides obtained from these acids melted at 113°.

† Yield not sufficient for analysis.

The data secured from the unsaturated acids in Fraction 30 indicate the presence of an unsaturated acid of greater molecular weight than those of the C_{18} series.

Separation of Unsaturated Fatty Acids into Two Fractions by Barium Soap-Benzene Method—Some of the unsaturated acids prepared by the lead soap-ether separation, after analysis, were subjected to the barium soap-benzene separation. The data secured are given in Table III.

As a clean cut separation of oleic acid was not obtained by one application of the method, the acids obtained from the barium soaps which were insoluble in benzene were subjected to two more repeated applications of this method of separation and as a result an acid was finally obtained which, after distillation at 2 mm. pressure, had a molecular weight of 281.4 and an iodine number of 89.9. This substance was very pure oleic acid.

The figures in the last two columns of Table III, which were secured by an examination of the acids obtained from the barium soaps which were soluble in benzene, indicate the presence of linoleic acid. When these acids were brominated in cold ether and the insoluble polybromides removed, the subsequent addition of a large volume of petroleum ether gave tetrabromides which in each case were found to melt at 113°, uncorrected. The tetrabromide of linoleic acid is said to melt at 113–115°. The successful isolation of this tetrabromide from the fat of human milk is a marked contrast to the negative results obtained with cow's milk fat as previously reported (1).

TABLE IV

Polybromide Numbers for Several Fractions and Calculated Amounts of Highly Unsaturated Acids Present

Fraction No.	Weight of fractions	Polybromide No.	Calculated amount of methyl esters
	gm.		*gm.*
23	112.17	Trace	
24	138.33	0.11	0.1951
25	244.17	0.29	0.9078
26	145.58	2.28	4.2554
27	12.17	4.17	0.6506
28	4.42	4.42	0.2505
29	4.25	9.07	0.4942
30	10.75	11.32	1.5590
Total amount of methyl ester per 1000 gm. fat, *gm.*			8.3126
Esters as a C_{22} acid, *gm.*			7.972
Highly unsaturated acid calculated as a C_{22} acid, *per cent*			0.797

Presence of Highly Unsaturated Acids—Because the polybromide number is an index of the presence of highly unsaturated fatty acids (2), these numbers were determined and are given in Table IV. Brown (3) has given a factor for calculating the amount of highly unsaturated acid present in a fat from the polybromide number of its methyl ester. These calculations have been made for several of the fractions and the results will be found in the last column of Table IV. It will be noticed that a distinct maximum for highly unsaturated acids appears in Fraction 26 and again in Fraction 30, indicating the presence of at least two of these acids. The ester polybromides from Fractions 25 to 28 melted with decomposition at 241–242°, while those from Fractions 29 and 30 did not melt. Methyl octobromoarachidate is said to melt at 228–231°. It would seem, therefore, that the polybromides from Fractions 25 to 28, which gave an indistinct melting point, were mixtures of methyl octobromoarachidate and a polybromide of the methyl ester of an acid of the C_{22} or higher series. The total amount of these highly unsaturated acids as calculated was found to be 0.792 per cent of the original fat.

TABLE V

Saturated Acids after Crystallization from Acetone

Fraction No.	Mol. wt.
23	257
24	264
25	283
26	284
27	294
28	*
29	*
30	339

* Yield not sufficient for analysis.

Saturated Acids of High Molecular Weight—The saturated acids obtained from several of the higher fractions by the lead soap-ether separation were crystallized twice from acetone to free them from traces of unsaturated acids, and the last trace of acetone was removed from the free acids by heating under diminished pressure. The molecular weights of these acids will be found in Table V. It will be noted that Fraction 30 gave acids with a molecular weight of 339, indicating the presence of a C_{22} saturated acid.

SUMMARY

1. 1000 gm. of the fat from mixed samples of milk from women have been converted to methyl esters; and these esters have been

fractionated into thirty fractions that were subjected to careful examination.

2. A maximum was obtained at the lauric acid point which is contrary to the findings for cow's milk fat, and from the highest fraction there was isolated a mixture of saturated acids of greater molecular weight than that of stearic acid.

3. Curves have been plotted based upon the total iodine absorption values of each of the fractions, and by means of these curves the presence of decenoic ($C_{10}H_{18}O_2$), tetradecenoic ($C_{14}H_{26}O_2$), hexadecenoic ($C_{16}H_{30}O_2$), oleic ($C_{18}H_{34}O_2$), and linoleic ($C_{18}H_{32}O_2$) acids was predicted, and the presence of all but decenoic acid was established.

4. There is evidence of the occurrence of unsaturated acids with greater molecular weights than those of the C_{18} series, and at least two highly unsaturated acids of the arachidonic acid type were shown to be present.

The author is greatly indebted to Professor J. B. Brown of the Department of Physiological Chemistry for his valuable suggestions and help during the conduct of this investigation, and to Dr. C. S. Smith, Chairman of the department, for encouragement and advice.

BIBLIOGRAPHY

1. Bosworth, A. W., and Brown, J. B., *J. Biol. Chem.*, **103**, 115 (1933).
2. Brown, J. B., and Sutton, T. S., *J. Dairy Sc.*, **14**, 125 (1931).
3. Brown, J. B., *J. Biol. Chem.*, **80**, 455 (1928).

THE DISTRIBUTION OF IRON IN TISSUES, PARTICULARLY LIVER, DURING PEPTIC DIGESTION AND AUTOLYSIS

BY W. D. MCFARLANE

(*From the Department of Biochemistry, University of Alberta, Edmonton, Canada*)

(Received for publication, April 30, 1934)

A large proportion of the iron in thoroughly perfused liver is in protein combination in so far as it is removable from solution by precipitation with trichloroacetic acid. Further, as experiments to be described herein show, about one-half of the iron contained in this trichloroacetic acid precipitate reacts with bipyridine in the presence of a reducing agent and must therefore be in some form of chemical combination other than that of the iron in hematin.

Aside from the observations which have been made on the protein vitellin, little is known of the chemical nature of iron-containing compounds of the above type. The literature in this regard has been reviewed by Jukes and Kay (1) and by McFarlane (2). A considerable part of the total iron in yeast is precipitated by trichloroacetic acid which, Coolidge (3) has concluded, from electrode potential measurements, is contained in an iron-protein complex.

Prolonged digestion of egg yolk protein by gastric juice was found by Miescher (4) to split off an iron-containing insoluble product which Bunge (5) later named hematogen. Hugounenq and Morel (6) believed hematogen to be a complex substance, a type of conjugated protein in which an iron-containing pigment is the prosthetic group, combined with a protein derivative. During the peptic digestion of blood no perceptible cleavage of iron from hemoglobin takes place and, further, salts of Fe^{++} and Fe^{+++} added to such a digest can be recovered quantitatively (Barkan (7)). In a study of the digestion by pepsin of artificially prepared iron albuminate, Mascherpa (8) has shown that the chemical state of the iron is altered, in that it is rendered dialyzable and easily detected.

The experiments recorded in this paper are primarily concerned with the study of the effects of proteolysis and autoproteolysis on the iron-containing components of tissues, precipitated by trichloroacetic acid, and particularly on those present in liver tissue.

EXPERIMENTAL

Distribution of Iron and Copper in Adult Rat Livers—In the following experiments entire rat livers, immediately following excision were thoroughly perfused with iron- and copper-free isotonic saline and reduced to a pulp in a mortar. Weighed portions of the liver pulp were taken without delay for the various analyses. One portion was dried to constant weight at 100°, the dry weight determined, and the sample ashed. The estimations of the total iron and copper content of the liver were made on aliquots of a solution of the ash. Iron was determined colorimetrically as ferric thiocyanate and copper by means of the carbamate reagent (McFarlane (9)).

Trichloroacetic acid precipitation of the liver, and the determination of iron and copper in the filtrate therefrom, was carried out as follows: Weighed portions of rat liver pulp (1 to 1.5 gm.) were pulverized in a small mortar with 10 cc. of 10 per cent trichloroacetic acid and a small amount of quartz sand.[1] The suspension was transferred to a 15 cc. centrifuge tube, two 2 cc. portions of 5 per cent trichloroacetic acid being used to rinse the mortar and pestle. The clear yellow-colored centrifugate was decanted into a 25 cc. volumetric flask. The protein residue was washed twice with 5 cc. portions of 5 per cent trichloroacetic acid, the washings added to the contents of the volumetric flask, and the solution made up to volume with distilled water.

The amount of iron in the trichloroacetic acid extract which reacted directly with potassium thiocyanate was determined as follows: A suitable aliquot (3 to 5 cc.) was measured into a 25 cc. glass-stoppered cylinder, 1 drop of superoxol was added, and after mixing the solution was diluted to 10 cc. with distilled water. 1 cc. of 40 per cent potassium thiocyanate solution was added, the ferric thiocyanate extracted with 2 cc. of isoamyl alcohol, and the final colorimetry carried out by the procedure already referred to (9). The iron content of separate aliquots of the above solution was also determined after ashing and after wet digestion with a sulfuric-perchloric acid mixture (McFarlane (10)).

---

[1] The quartz sand was previously extracted with boiling concentrated HCl for several hours, filtered, and washed with water on a Jena sintered glass filter. The trichloroacetic acid was redistilled and contained no iron or copper.

A 5 cc. aliquot of the trichloroacetic acid filtrate was precipitated in a centrifuge tube with 2 cc. of saturated normal lead acetate. After centrifuging and discarding the supernatant fluid, the small white precipitate was dissolved in a minimum amount of 10 per cent trichloroacetic acid and reprecipitated by normal lead acetate. The precipitate was finally suspended in 0.1 N H_2SO_4 and decomposed with H_2S. This precipitate contains iron which reacts directly, in acid solution, with potassium thiocyanate, the amount of which was determined as above.

TABLE I

Distribution of Iron and Copper in Adult Rat Livers

The results are expressed in mg. per 100 gm. of dry tissue.

Body weight	Iron						Copper	
	Total	Non-hematin	Trichloroacetic acid filtrate				Total	Trichloroacetic acid filtrate
			Total Fe			Lead acetate precipitate		
			Direct*	Ashing	Wet digestion			
gm.								
	69	31					1.56	1.71
179	65	39					1.58	1.52
178	63	27					1.66	1.55
300	43	22	8.7	9.5			1.30	1.39
315	52	25	8.5	7.8	8.0	4.6	1.19	1.22
280	49	22	9.0	9.6	8.9	5.2		
	55		8.2		8.0	4.2		

* Determined by direct colorimetry in acid solution after oxidizing with hydrogen peroxide.

The entire trichloroacetic acid extract from 2.0 to 2.5 gm. of liver pulp was taken for the estimation of copper. The determinations were made by direct colorimetry with the carbamate reagent (9), without previous ashing or wet digestion.

The amount of iron in the liver in "non-hematin" combination was determined as follows: A weighed quantity of liver pulp was ground in a mortar with 2 cc. of acetate buffer (pH 4.0) and a small amount of quartz sand. The suspension was transferred to a 25 cc. Erlenmeyer flask with the aid of 10 cc. of acetate buffer. After adding 0.5 cc. of bipyridine solution and a small amount of iron-free solid sodium hydrosulfite, sulfur dioxide was bubbled through the suspension for about 5 minutes. The ferrous bipyridine color developed very slowly, reaching maximum intensity only after 5 hours. After this time the material was filtered into a 100 cc. volumetric flask and the precipitate washed on the filter with 30 per cent alcohol until the volume of the filtrate was 100 cc. In some cases a white turbidity was removed by centrifuging, before the color intensity was matched against color standards (Hill (11)).

The results are summarized in Table I, concerning which the following observations may be made.

Approximately one-half of the total iron of liver reacts directly with bipyridine, while less than 50 per cent of this non-hematin iron appears in the trichloroacetic acid filtrate. The iron in this filtrate reacts directly and quantitatively with potassium thiocyanate in acid solution in the presence of hydrogen peroxide. A fairly constant proportion of the iron in the trichloroacetic acid filtrate is contained in an organic fraction precipitated by normal lead acetate. It would appear that all of the copper in adult rat liver is to be found in the trichloroacetic acid filtrate and is in ionic form, as evidenced by its direct reaction with sodium diethyldithiocarbamate.

Similar experiments with perfused rat spleen have revealed that all the iron extracted by trichloroacetic acid (0.0023 gm. of Fe per 100 gm. of wet tissue) also reacts with potassium thiocyanate in acid solution containing hydrogen peroxide.

Autolysis and Peptic Digestion of Rat Liver and Muscle Tissue

The entire livers of adult rats were thoroughly perfused with 0.9 per cent sodium chloride and reduced to a pulp in a mortar. Weighed portions, about 5 gm., were autolyzed under the conditions recommended by Herron and McEllroy (12) by adding 20 cc. of 0.02 N HCl and 2 cc. of chloroform and incubating at 37°.

Peptic digestion was carried out by suspending similar amounts of the liver pulp in 25 cc. portions of a 0.2 per cent solution of

U.S.P. pepsin in 0.1 N HCl. The digestion was carried out at 37° in the presence of 2 cc. of chloroform. The thigh and leg muscles of adult rats were prepared for, and subjected to, autolysis and peptic digestion as with the liver.

After varying periods of time, each autolysate or peptic digest was poured into a 50 cc. centrifuge tube containing 10 cc. of a 20 per cent trichloroacetic acid solution. The centrifugate was decanted into a 50 cc. volumetric flask and the protein residue washed twice with 5 cc. portions of 5 per cent trichloroacetic acid. The combined centrifugates were made up to volume with distilled water.

TABLE II

Iron Content of Trichloroacetic Acid Filtrates of Autolysates and Peptic Digests of Adult Rat Liver and Muscle Tissue

The results are expressed in mg. of Fe per 100 gm. of wet tissue.

Tissue	Autolysis				Peptic digestion			
	Time	Total (ashing)	Direct	Lead precipitate	Time	Total (ashing)	Direct	Lead precipitate
	hrs.				*hrs.*			
Liver	0	1.12	1.06	0.55	0	1.4	1.1	0.6
	20	4.90	4.60	1.37	3	5.9		6.0
	66	2.18	2.01	0.96	20	13.7	12.8	8.4
	98	1.16	0.94	0.49	96	12.0	11.8	7.1
Muscle	0				0	1.58	1.77	0.87
	24	1.43	1.34	1.17	2	2.81	2.84	1.16
	76	2.19	2.17	0.66	10	3.00	2.91	0.98
	173	1.90	2.16	0.59	149	3.22	3.10	

The iron and copper analyses of these solutions were carried out as already described. The following points arise from a consideration of the results in Table II. (*a*) During the course of the proteolysis of liver and muscle tissue by pepsin, the amount of iron in the protein-free filtrate of the hydrolysate markedly increases. (*b*) The early stages of the autolysis of these tissues is also accompanied by an increase in the iron content of the protein-free filtrate, which is, however, of much smaller magnitude. As autolysis continues there is an apparent decline in the amount of iron appearing in the trichloroacetic acid filtrate. This latter phenomenon is particularly marked in the case of the liver autolysate. (*c*) These fluctuations in the total iron content of the protein-free filtrates of the autolysates and hydrolysates are paralleled by changes of similar degree in the amount of iron which is precipitable by normal lead acetate. The amount of copper in the protein-free filtrates of the peptic digests and autolysates of liver remained relatively constant, varying only within limits (0.53 to 0.57 mg. per 100 gm. of wet tissue) attributable to experimental error. In the experiments with muscle tissue no copper analyses were made.

The results of these autolysis experiments were entirely unexpected and are not easy to explain. The following experiments completely confirm these findings and present some additional observations.

Autolysis and Peptic Digestion of Fetal Calf Liver

In the following experiments fetal calf liver was used because it could be easily reduced to a liquid consistency, which permitted our measuring the samples by pipette instead of by weighing. The entire liver was perfused with isotonic saline as thoroughly as possible and the surface roughly dried by pressing between filter papers. The tissue, after mincing, was squeezed repeatedly through cheese-cloth by the use of a press. Portions of this liver pulp were digested either with pepsin, gastric juice, or autoproteolytic enzymes, and at varying times during the course of proteolysis flasks were removed from the 37° incubator and their contents precipitated with trichloroacetic acid.

The following is a protocol of the experiments.

Autolysis—Twelve flasks were used, each containing 10 cc. of liver pulp, 40 cc. of 0.02 N HCl, and 2 cc. of chloroform. The contents of one flask (0 hours) were immediately precipitated by pouring into a 100 cc. centrifuge tube containing 10 cc. of 20 per cent trichloroacetic acid. The flask was rinsed out with 5 cc. of 5 per cent trichloroacetic acid and the rinsings were used to wash the protein precipitate. The washing was repeated, the precipitate being thoroughly stirred each time with a glass rod. The combined centrifugates were brought to a volume of 100 cc. Before the remaining eleven flasks were placed in the incubator, the contents of three of them were further treated as follows: H_2S was bubbled through one of the liver suspensions for 5 minutes, 0.3 mg. of Cu ($CuSO_4$) was added to another, and 3.0 mg. of Cu to the third. After varying periods of time up to 10 days, flasks were removed from the incubator and their contents precipitated with trichloroacetic acid as stated above.

Peptic Digestion—Ten flasks were used, each containing 10 cc. of liver pulp, 50 cc. of a 0.2 per cent solution of pepsin (U.S.P.) in 0.1 N HCl, and 2 cc. of chloroform. The contents of one flask were immediately precipitated with trichloroacetic acid as above. Again, the contents of three of the remaining flasks were further treated with H_2S or copper sulfate in the manner described above. These nine flasks were incubated at 37° for varying periods of time up to 145 hours.

Digestion with Gastric Juice—Four flasks were used, each containing 10 cc. of liver pulp, 50 cc. of a solution containing 60 cc. of filtered normal gastric juice and 120 cc. of 0.1 N HCl in a total volume of 200 cc., and 2 cc. of chloroform. The contents of each flask were approximately pH 1.5. Digestion was allowed to proceed at 37° for varying periods of time up to 143 hours.

The iron and nitrogen content of each protein-free filtrate was determined. Direct colorimetry with potassium thiocyanate, after oxidizing with hydrogen peroxide, was employed. The results were checked, both colorimetrically and by titanium titration, by a number of determinations made after ashing. The inorganic phosphorus content of the protein-free filtrate of the autolysates was determined by the method of Fiske and Subbarow (13).

The results of these analyses are shown in Figs. 1 and 2. The digests to which copper was added and those through which H_2S was passed gave the results shown in Table III.

Proteolysis of liver by pepsin or gastric juice (Fig. 1) is accompanied by a marked increase in the iron content of the protein-free filtrate over that produced during autolysis. While the amount of iron liberated by pepsin reaches a maximum only after about 80 hours, and remains at this high level during prolonged digestion, that brought about by autolysis reaches a maximum in approximately 20 hours, and then begins to decline. After autolyzing for 10 days, the iron content of the protein-free filtrate is only about one-fourth of that in the undigested liver. These results definitely confirm the previous observations made in experiments with rat liver.

From a comparison of the curves in Figs. 1 and 2, there would appear to be no correlation between the increase in non-protein nitrogen and the increase in inorganic iron during pepsin action. On the other hand there is evidence that the liberation of iron during autolysis is brought about by the same reaction as that responsible for the increase in non-protein nitrogen and inorganic phosphorus. These changes are presumably due to cathepsin activity. This is further borne out by the fact that the addition of copper (Table III) not only inhibits the production of non-protein nitrogenous compounds and inorganic phosphorus but also retards the liberation of iron, although to a less marked degree; while all three phases of the reaction are accelerated by the presence of hydrogen sulfide. Stern (14) has found that cathepsin in calf liver and spleen is activated by iron but is inhibited by copper.

TABLE III

Analysis of Trichloroacetic Acid Filtrates of Specially Treated Autolysates and Peptic Digests

Digestion time	Treatment	Iron	Phosphorus	Total nitrogen
hrs.		*mg. per 100 gm. liver*	*mg. per 100 gm. liver*	*per cent*
43	Autolysate untreated	13.8	62	0.78
43	" + 0.3 mg. Cu	10.4	41	0.40
43	" + 3.0 " "	10.9	47	0.46
43	" + H_2S	70.5	76	0.94
11	Peptic digest untreated	26.4		1.10
11	" " + 0.3 mg. Cu	25.2		1.07
11	" " + 3.0 " "	26.1		1.13
11	" " + H_2S	72.4		1.14

The recombination of iron with protein (?) is evidently brought about by some mechanism other than that responsible for its cleavage, since the non-protein nitrogen and the inorganic phosphorus do not show any similar decline. The addition of copper to the peptic digest was without effect, but the presence of H_2S

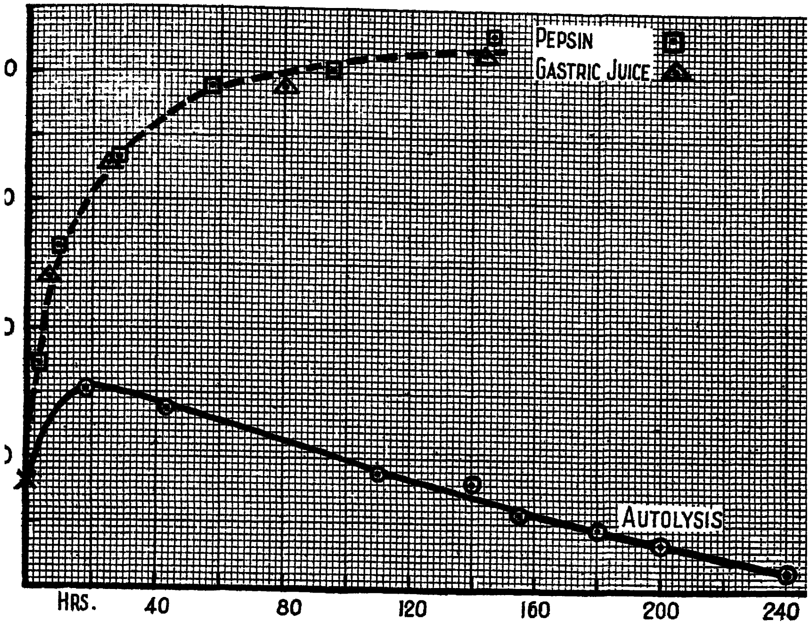


FIG. 1. The effect of autolysis and peptic digestion on the iron content of the protein-free filtrate from fetal calf liver.

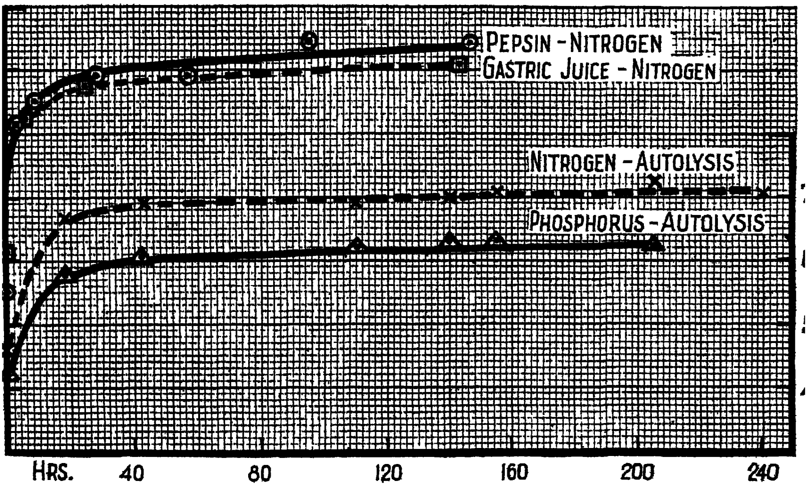


FIG. 2. The effect of autolysis and peptic digestion on the nitrogen and inorganic phosphorus content of the protein-free filtrate from fetal calf liver.

brought about a marked increase in the iron content of the protein-free filtrate over that produced, in equal time, by pepsin. H_2S is known to activate plant proteases (15).

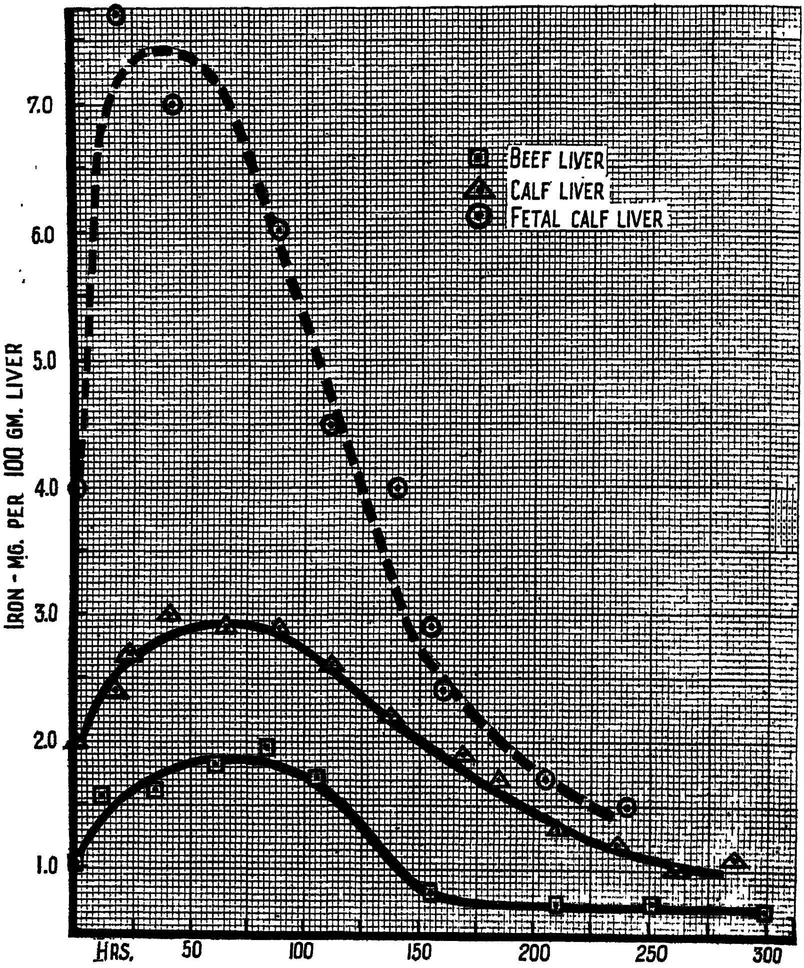


FIG. 3. The effect of autolysis on the iron content of the protein-free filtrate of liver from different sources. The values for fetal calf liver are divided by 2.

Similar autolysis experiments have been conducted with calf liver and beef liver. The curves in Fig. 3 compare the results obtained with those already recorded for fetal calf liver. It will be seen that in using fetal calf liver in our first experiments we have accidentally chosen a source of liver most admirably suitable for demonstrating the changes in iron distribution accompanying autolysis. These results will be referred to again in connection with later experiments.

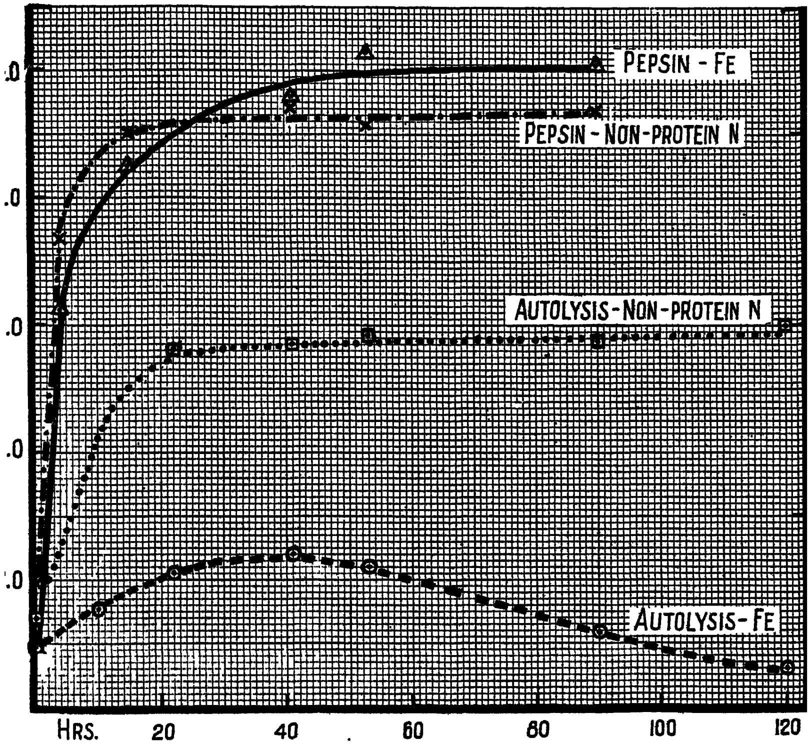


FIG. 4. The effect of autolysis and peptic digestion on the iron and nitrogen content of the protein-free filtrate of beef spleen.

That the autolysis and proteolysis of beef spleen are accompanied by changes in the iron content of the protein-free filtrate similar to those found with liver is shown in Fig. 4. In the peptic digestion of spleen tissue, however, the reactions involving the liberation of inorganic iron and the increase in non-protein nitrogen seem to be more closely related.

Some Factors Affecting These Changes Accompanying Autolysis of Liver

Influence of Previous Treatment with H_2S—Experiments with anemic rats conducted by a number of workers (Cunningham (16), Josephs (17), Elvehjem and Sherman (18)) have revealed that copper catalyzes (*in vivo*) the conversion of inorganic iron into organic iron compounds in the liver. We have endeavored to demonstrate that copper has a similar action in these experiments, retarding the decomposition of iron compounds in the early stages and accelerating the recombination of iron with organic substances in the later stages of autolysis.

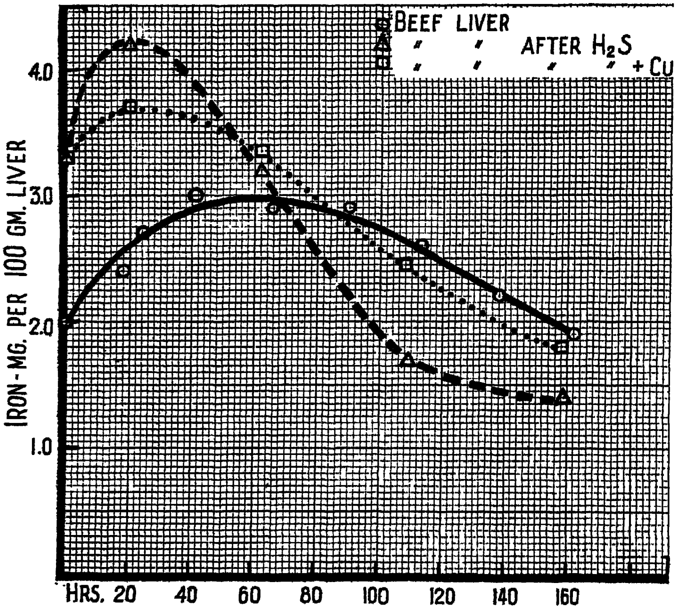


FIG. 5. The effect of previous treatment with hydrogen sulfide on the changes in the iron content of the protein-free filtrate during autolysis.

Preliminary experiments in this regard (Table III) showed a marked acceleration in the liberation of inorganic iron brought about by H_2S, and a slight retardation of the process by added copper. The activation by H_2S may possibly be due to the removal of the inhibitory action of copper. Krebs (19) has suggested that the activation of cathepsin by hydrosulfide compounds is due to the removal of inhibiting heavy metals by means of complex formation. The effects of adding copper to the autolysates can only be demonstrated satisfactorily when the copper already present in the liver has been removed. Since it appears that this copper is present in ionic form, it should be possible to effect its conversion into cupric sulfide and thus prevent its action. This was attempted by preliminary treatment of the tissue with H_2S. After removing the H_2S by distilling *in vacuo*, the autolysis was carried out with and without added copper as follows.

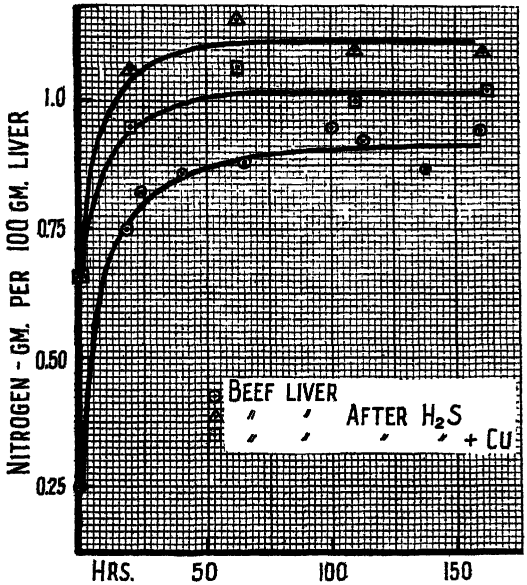


FIG. 6. The influence of previous treatment with hydrogen sulfide on the changes in the non-protein nitrogen during autolysis.

To 100 gm. of beef liver pulp were added 200 cc. of 0.05 N HCl and the suspension was saturated with H_2S for 30 minutes. The H_2S was then removed by distilling *in vacuo* at 40°, and the material finally brought to a volume of 505 cc. A 50 cc. aliquot was placed in each of ten flasks. To five of the flasks was added 1 cc.

of a copper sulfate solution containing 12 mg. of Cu per cc., and to the remaining five flasks an equal amount of water was added. After adding 2 cc. of chloroform to each flask, the autolysis experiment was conducted in the manner described for previous experiments.

The results of these experiments are shown in Figs. 5 and 6. The first value recorded on each curve is that determined immediately following the removal of H_2S. The results with the same beef liver untreated are those of a previous experiment and are recorded for comparison.

The initial stages of the autolysis of liver tissue, which had been previously treated with H_2S, are characterized by a marked acceleration of the reactions involving the splitting of iron from organic combination (Fig. 5) and the production of non-protein nitrogen (Fig. 6). These changes are inhibited by copper. The magnitude and the velocity of the reverse reaction between inorganic iron and some substance precipitated by trichloroacetic acid, which takes place during prolonged autolysis, are also greatly increased. Contrary to what we had anticipated, copper also appears to retard this change.

We did not succeed, however, in converting all the copper in the liver pulp into cupric sulfide by the above procedure. The copper content of the liver pulp as determined after ashing the protein-free filtrate was 7.1 mg. per kilo of wet tissue. This value was reduced to 1.4 mg. per kilo by treatment with H_2S.

Autolysis in Presence of H_2S—In the preceding experiment it was found that during the 30 minutes that the liver tissue was in contact with H_2S a very rapid cleavage of the iron- and nitrogen-containing fractions took place. It was decided to repeat the experiment but to keep the liver suspension saturated with H_2S throughout autolysis. The results, shown in Fig. 7, furnish additional proof that the same reaction is responsible for the increase in both nitrogen and iron in the trichloroacetic acid filtrate. This reaction is presumably promoted by cathepsin and is strongly activated by hydrogen sulfide.

It will be seen (Fig. 7) that in the duration of this experiment no resynthesis of organic iron compounds has taken place. The control curve is a portion, the first 80 hours, of the complete curve for autolyzing calf liver (Fig. 3) in which the decline in inorganic iron did not commence until autolysis had proceeded about 100 hours. Later experiments show that the time at which the decline commences is correlated with the peak in the inorganic iron produced during the initial stages of autolysis and that the amount of iron recombining with organic substances during prolonged autolysis is roughly proportional to the amount of inorganic iron produced by proteolysis. From this standpoint, therefore, the same decline in the inorganic iron was to be expected in this experiment as that shown in the preceding experiment with H_2S (Fig. 5). Since the amount of iron liberated by proteolysis in the present experiment remains at its maximum up to 80 hours autolysis, it would appear that the recombination of iron with organic substances is prevented by the presence of H_2S.

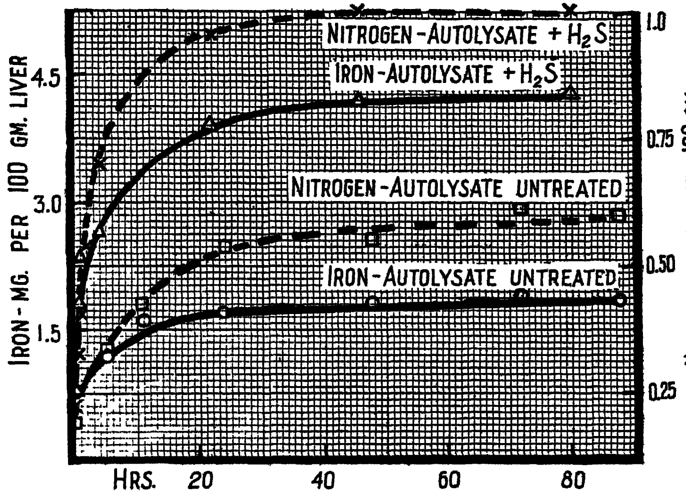


FIG. 7. The effect of the presence of hydrogen sulfide on the iron and nitrogen content of the protein-free filtrate of autolyzing calf liver.

Prior to treatment with H_2S, the calf liver contained 7.8 mg. of copper per kilo of wet tissue as determined in the trichloroacetic acid filtrate. At the conclusion of the experiment, the copper content was reduced to 1.9 mg. per kilo of wet tissue. A portion of the copper present in the liver is not precipitable by H_2S in acid solution.

Autolysis with Added Iron—We have carried out some experiments to determine the effects of added ferric chloride on the changes in the distribution of the iron in liver during autolysis. A number of liver suspensions were prepared, each containing 50 cc. of the same calf liver pulp to which were added varying amounts of a solution of ferric chloride in 0.05 N HCl and an amount of 0.05

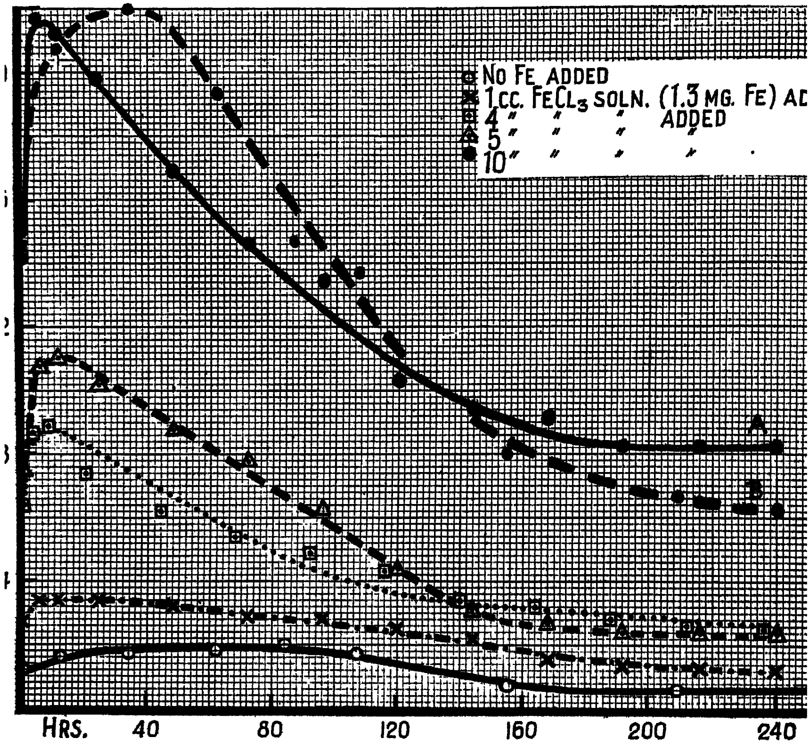


FIG. 8. The effect of added iron on the changes in the iron content of the protein-free filtrate of autolyzing calf liver. Experiments A and B were performed with two different samples of calf liver to which different amounts of HCl were added.

N HCl to make a total of 100 cc. of 0.05 N HCl. To each flask were added 10 cc. of chloroform. During the course of autolysis 10 cc. aliquots were removed from each flask and the iron content of the trichloroacetic acid filtrate therefrom determined.

The results, expressed in the form of graphs in Fig. 8, afford

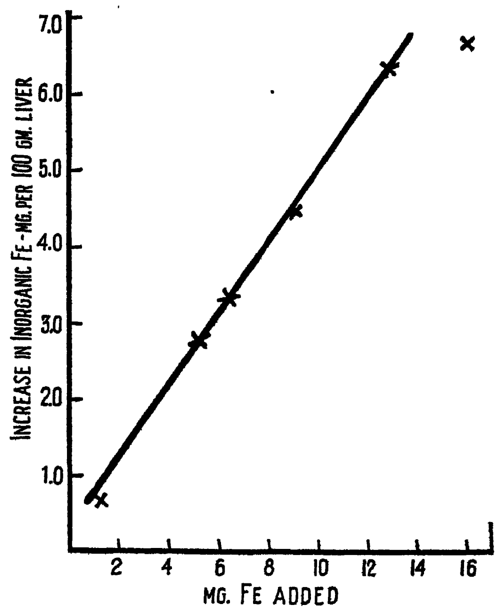


FIG. 9. The proportionality between the increase in inorganic iron after 5 hours autolysis and the amount of iron added to the autolysates.

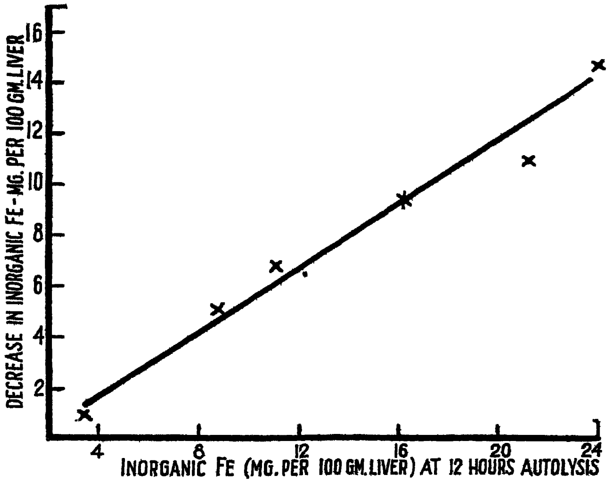


FIG. 10. The relationship between the decrease in inorganic iron from 12 to 120 hours autolysis and the amount of inorganic iron liberated during the first 12 hours of autolysis.

unquestionable evidence that the magnitude of the changes in the distribution of iron during liver autolysis depends upon the initial concentration of iron. The quantitative relationship between these changes, *i.e.* the increase in inorganic iron in the early stages of autolysis and the subsequent recombination of iron with organic substance during prolonged autolysis, and the one variable (the amount of iron present at the commencement of autolysis) is shown in Figs. 9 and 10. Two of the values plotted in each of these graphs are calculated from the data of two experiments with added iron (9.2 and 16.1 mg. of Fe, respectively) which are not recorded in Fig. 8. The difference in the results with liver from various sources (Fig. 3) is now explainable as being due to the amount of inorganic iron which they contain.

In Experiment B (Fig. 8) the autolysate was prepared by adding to 50 cc. of liver pulp, 250 cc. of 0.02 N HCl (containing 13 mg. of Fe as $FeCl_3$) and 10 cc. of chloroform. During the course of autolysis 25 cc. aliquots were removed for analysis. The iron analysis of the trichloroacetic acid filtrates (Experiment B, Fig. 8) when contrasted with Experiment A illustrates the variations in the results to be expected from modifications of the experimental conditions. Two different samples of calf liver were used in preparing these autolysates and different amounts of acid were added to each. In Experiment A the equivalent of 3.1 cc. of N HCl was added and in Experiment B the equivalent of 1.6 cc. of N HCl.

We have not yet determined the influence of pH on the changes in the distribution of iron during autolysis. The actual change in pH during the course of the autolysis of beef liver (Fig. 3) has, however, been determined with the use of the quinhydrone electrode. The initial pH 4.6 changed to pH 4.4 after 3 days autolysis and to pH 4.3 at the conclusion of the experiment.

The effects of adding varying amounts of a dried liver extract powder[2] to autolysates of the same composition as those of Experiment B have also been studied. The liver extract affected the results only by virtue of the iron which it contributed. The iron analyses of the trichloroacetic acid filtrates in one of these experiments (Experiment C), in which 3.48 gm. of liver extract powder were added, are recorded in Table IV.

---

[2] Connaught Laboratories.

Iron Content of Trichloroacetic Acid Precipitates from Autolyzing Liver

The trichloroacetic acid precipitates in Experiment B were suspended in water, filtered, and thoroughly washed with water on the filter paper. The residues were dried on the filter papers at 100°. The iron content of a portion of each dried residue was determined after digesting with perchloric-sulfuric acid mixture.

With the use of the trichloroacetic acid precipitates retained from Experiment C, the solubility of the iron compounds therein, in warm 80 per cent alcohol, was determined as follows: Each trichloroacetic acid precipitate was suspended in about 75 cc. of of water and dissolved by adding 2 cc. of N NaOH. The solution was heated on a boiling water bath for 30 minutes, cooled, and reprecipitated by adjusting to pH 6.5 (thymol blue) with 0.4 cc. of N HCl. After evaporating to a volume of about 15 cc., the solution was made 80 per cent alcoholic by adding absolute alcohol. The precipitate was separated by centrifuging and the clear supernatant liquid decanted. The alcohol extracts from the different

TABLE IV

Distribution of Iron between Trichloroacetic Acid Precipitate and Filtrate of Autolyzing Calf Liver

The results are expressed in mg. of Fe per 25 cc. of autolysate.

Duration of autolysis	Experiment B				Experiment C					
	Precipitate		Filtrate total	Total Fe	Trichloroacetic acid filtrate	Trichloroacetic acid precipitate reprecipitated from 80 per cent alcohol				
						Precipitate			Filtrate total	Total Fe
	Total	Per cent				Dry weight	Total	Per cent		
hrs.						*mg.*				
0	0.47	0.097	0.59	1.06	0.900	401.8	0.418	0.104	0.009	1.327
12	0.27	0.083	0.87	1.14	1.090	288.8	0.220	0.076	0.007	1.313
34	0.21	0.076	0.92	1.13	0.946	283.4	0.344	0.121	0.009	1.299
62	0.34	0.106	0.80	1.14	0.830	232.9	0.357	0.153	0.004	1.191
84	0.47	0.144	0.61	1.08	0.784	274.4	0.440	0.160	0.007	1.231
107	0.49	0.151	0.57	1.06	0.666	255.8	0.512	0.200	0.007	1.180
155	0.59	0.180	0.33	0.92	0.620	262.5	0.580	0.221	0.022	1.222
209	0.72	0.213	0.24	0.97						
240	0.80	0.250	0.21	1.01	0.548	259.5	0.727	0.280	0.015	1.290

trichloroacetic acid precipitates varied in color from a pale yellow (0 hours autolysis) to a golden brown color (107 hours autolysis). The precipitate was washed by stirring with 20 cc. of 80 per cent alcohol in a water bath at 50° for approximately 10 to 15 minutes. Four such washings were necessary to extract all of the yellow pigment. The combined alcohol extracts were evaporated to a small volume and the iron content determined after digesting with perchloric-sulfuric acid mixture.

The alcohol precipitate was dried at 100° for 24 hours, weighed, and samples taken for iron determinations as before.

The results of these analyses are shown in Table IV. It may be concluded from these results that the iron which disappears from the trichloroacetic acid filtrate during prolonged autolysis is to be found in combination with substances also insoluble in warm 80 per cent alcohol. Although the alcohol extracts contain a considerable amount of material, including a yellow pigment the amount of which increases with the duration of the autolysis up to 107 hours, they contain only a negligible proportion of the total iron.

Nature of Iron-Containing Substance Precipitated by Trichloroacetic Acid

The result of these studies which calls for special comment is the finding that, during prolonged autolysis of liver and spleen, synthesis of an organic iron compound takes place, presumably by the interaction of inorganic iron and some primary product of autoproteolysis. In the following fractionation of liver we have separated a substance, proteose in nature and rich in iron, with properties which suggest its identity with the iron-containing substance formed during autolysis.

Beef liver (7 kilos) was fractionated by the procedure of McHenry, MacLean, and Best (20). The material precipitated from 95 per cent alcohol after removal of proteins, and which constitutes their "liver extract," still contains substances precipitable by trichloroacetic acid. This fraction was separated quantitatively and twice reprecipitated by dissolving in the minimum amount of 0.1 N NaOH and precipitating from a 3.5 per cent trichloroacetic acid solution. The precipitate was again dissolved in 0.1 N NaOH and an equal volume of 5 per cent H_2SO_4 added. Precipitation with mercuric sulfate from ether-alcohol solution according to Felix and Frühwein (21) was next carried out. The mercury precipitate, after washing, was decomposed with H_2S and the sulfuric acid removed with baryta. Evaporating the filtrate to dryness yielded 0.23 gm. of a yellow-colored residue containing 0.33 per cent Fe and 16.3 per cent N. This fraction gave a strong biuret reaction, was insoluble in 80 per cent alcohol, and was extremely resistant to low temperature ashing with HNO_3 (McFarlane (9)). We have not examined this material further and have still to determine whether its amount is increased by autolysis. The filtrate after trichloroacetic acid precipitation contains material precipitated by tannic acid but which, however, contains no iron.

SUMMARY

The distribution of iron and copper in adult rat liver has been studied with the following results. (*a*) Approximately one-half (43 to 60 per cent) of the iron in perfused rat liver tissue is in non-hematin form in so far as it reacts with bipyridine after reduction with sodium hydrosulfite. (*b*) The total iron in a trichloroacetic acid filtrate reacts directly with potassium thiocyanate in acid solution after oxidation with hydrogen peroxide. The iron in this fraction accounts for only about 40 per cent of the non-hematin iron. (*c*) About 50 per cent of the total iron in the trichloroacetic acid filtrate is precipitated along with organic substances by normal lead acetate. (*d*) All of the copper in liver tissue is contained in the trichloroacetic acid filtrate and reacts directly with sodium diethyldithiocarbamate.

The digestion of fetal calf liver, adult rat liver and muscle tissue, and beef spleen by pepsin at about pH 2.0 produces a 5-fold increase in the iron content of the trichloroacetic acid filtrate. The value reaches a maximum in about 40 hours.

The autoproteolytic changes in liver and spleen at pH 4.5 also include the decomposition of organic iron-containing compounds, presumably iron proteinates. This decomposition is accelerated by H_2S and inhibited by copper. During prolonged autolysis there takes place a recombination of iron with organic substances (presumably proteose in nature), the magnitude of which is dependent upon the amount of iron liberated by proteolysis.

I desire to express my thanks to Professor G. Hunter for his helpful advice. I am also indebted to Mr. H. Tarver and Mr. A. McKeever for assistance with some of the analyses.

BIBLIOGRAPHY

1. Jukes, T. H., and Kay, H. D., *J. Nutrition*, **5**, 81 (1932).
2. McFarlane, W. D., *Biochem. J.*, **26**, 1061 (1932).
3. Coolidge, T. B., *J. Biol. Chem.*, **98**, 755 (1932).
4. Miescher, F., in Hoppe-Seyler, F., Medizinische-chemische Untersuchungen, Berlin, **4**, 502 (1870).
5. Bunge, G., *Z. physiol. Chem.*, **9**, 49 (1885).
6. Hugounenq, L., and Morel, A., *Compt. rend. Acad.*, **142**, 805 (1906).
7. Barkan, G., *Z. physiol. Chem.*, **148**, 124 (1925).
8. Mascherpa, P., *Boll. Soc. ital. biol. sper.*, **5**, 142 (1930).
9. McFarlane, W. D., *Biochem. J.*, **26**, 1022 (1932).
10. McFarlane, W. D., *Biochem. J.*, **26**, 1034 (1932).
11. Hill, R., *Proc. Roy. Soc. London, Series B*, **107**, 205 (1930).
12. Herron, W. F., and McEllroy, W. S., *Science*, **76**, 127 (1932).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
14. Stern, K. G., *Biochem. Z.*, **234**, 116 (1931).
15. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London and New York, 143 (1930).
16. Cunningham, I. J., *Biochem. J.*, **25**, 1267 (1931).
17. Josephs, H. W., *J. Biol. Chem.*, **96**, 559 (1932).
18. Elvehjem, C. A., and Sherman, W. C., *J. Biol. Chem.*, **98**, 309 (1932).
19. Krebs, H. A., *Biochem. Z.*, **220**, 269 (1930).
20. McHenry, E. W., MacLean, D. L., and Best, C. H., *Canad. Med. Assn. J.*, **26**, 397 (1932).
21. Felix, K., and Frühwein, H., *Z. physiol. Chem.*, **216**, 173 (1933).

THE BLOOD LIPIDS IN COMPLETELY DEPANCREATIZED DOGS MAINTAINED WITH INSULIN*

By I. L. CHAIKOFF and A. KAPLAN

(*From the Division of Physiology, University of California Medical School, Berkeley*)

(Received for publication, March 12, 1934)

Despite the fact that the completely depancreatized dog can now be maintained in good nutritional condition for long periods of time by means of insulin, certain pathological changes make their appearance in the tissues of these animals. In a previous communication it was shown that a large proportion of the depancreatized dogs kept in this laboratory for periods over a year developed cataractous involvements of the lenses, which varied from faint striations to diffuse opacification (1). The present report deals with the blood lipids of these animals. A marked disturbance was observed in these constituents of the blood. In addition to a general lowering of the lipid level, the esterified cholesterol fraction had either completely disappeared or had been greatly reduced in amount.

EXPERIMENTAL

Completely Depancreatized Dogs—Following complete pancreatectomy, the animals were fed twice daily a diet consisting of 225 gm. of lean meat, 70 gm. of sucrose, and 5 gm. of bone ash. Each dog also received a vitamin B concentrate prepared from rice bran (2). From the beginning of the experiment to September 1, 1933, vitamins A and D were supplied as the unsaponifiable portion of cod

---

* A preliminary report of this investigation appeared in *Proc. Soc. Exp. Biol. and Med.*, **31**, 149 (1933).

The expense of this investigation was defrayed in part by grants from the Research Board of the University of California, Berkeley, and from the Christine Breon Fund for Medical Research of the University of California Medical School, San Francisco. The insulin was generously donated by Eli Lilly and Company.

liver oil. On the latter date, however, raw cod liver oil was substituted as the source of these vitamins. The animals were injected with 8 units of insulin at each time of feeding. The 24 hour sample of urine always contained glucose in varying amounts from day to day. Throughout the period of observation indicated in Tables I and II, the animals were in a well nourished state and possessed ravenous appetites, immediately consuming all food presented. Ten depancreatized dogs are included in this study.

Normal Dogs—To obtain normal lipid values, ten normal animals were fed twice daily a mixture consisting of meat, sucrose, and bone ash. They also received the vitamins that were supplied to the depancreatized dogs. Two of the normal dogs were litter mates of animals that had been depancreatized. Thus dog N.A. was a litter mate of dog D. C., which had been depancreatized on September 1, 1931. Dogs D. B., D. G., and N. B. are also litter mates, the two former having been depancreatized and the latter retained as a normal control. The normal dogs were maintained on this diet for periods varying from 32 to 838 days. All normal animals used were in good nutritional condition and completely ingested all food in a very few minutes after it was served.

Sampling of Blood—The animals were fed at 8.00 a.m. and 4.00 p.m. daily, while the samples were taken, as a rule, between 7.00 and 8.00 a.m. on the days recorded. Hence, unless otherwise stated, examination of the blood was made between 15 and 16 hours after the ingestion of the last meal and the last injection of insulin. 10 cc. of whole blood taken from the femoral artery were pipetted with continuous stirring into a 125 cc. Erlenmeyer flask containing 40 cc. of 95 per cent alcohol. Analyses were begun as a rule immediately after the blood sample was taken; but when this was not possible the flasks were stoppered with tin-foil-covered corks and stored in the dark at $-1°$ until analyzed. The animals were accustomed to being handled and showed no excitement during removal of blood.

Extraction of Blood—The sides of the flask were scraped clean of adhering blood and washed down with amounts of 95 per cent ethyl alcohol and ethyl ether, sufficient to make the volume of solvent in the flask 75 cc. of a 3:1 alcohol-ether mixture. The mixture was refluxed in a water bath for 1 hour at approximately 55°, with vigorous rotation of the contents at intervals. A glass coil, sealed through the bottom of a small beaker, served both as condenser and cover for the flask. The coil was cooled by the passing of a stream of cold water through it. After cooling, the entire mixture was transferred quantitatively to a 100 cc. glass-stoppered volumetric flask. The contents were made up to volume at 20° and filtered through fat-free filter paper into a glass-stoppered flask. Inasmuch as alcohol, ether, and petroleum ether have relatively high coefficients of expansion, extracts were always made up to volume at 20° and brought back to that temperature when samples were removed for analyses.

Determination of Cholesterol (Free, Total, and Esterified)—Free cholesterol and total cholesterol were determined after the manner of Okey (3), with modifications suggested to us by Dr. Okey herself. The lipids were saponified with freshly prepared 2.5 per cent sodium ethylate instead of sodium hydroxide. After acidification the residue was completely extracted with petroleum ether. The successive portions of petroleum ether were filtered into a glass-stoppered volumetric flask and made up to volume. Aliquot portions were taken for determination of both total cholesterol and total fatty acids. Carbon dioxide was used instead of air as the agent for removing the last traces of solvent. The time of oxidation was extended to 35 minutes.

A modification of the acetone washing introduced by Yasuda (4) was used in the determination of free cholesterol. Following the evaporation of the alcohol, which results in the complete precipitation of the cholesterol digitonide, 5 cc. of acetone were added to the flask and the contents warmed to facilitate the disintegration of the residue. 15 cc. of ethyl ether were then added. Thereafter Okey's procedure was followed, except that after the fourth ether washing the asbestos pad was washed with a few cc. of acetone and sucked dry, and the remaining portions of acetone were then removed with a little cold water. In this way the last traces of ether were removed from the pad with a minimum of suction.

Esterified cholesterol was calculated as the difference between total and free cholesterol.

Phospholipid—The method of Bloor (5), with a few minor changes, was used. After the washing of the precipitated phospholipids with acetone, the precipitate was drained and dried in a current of carbon dioxide. The modifications of Page *et al.* (6)

were adopted in so far as the extraction process was concerned. The precipitated phospholipids were dissolved in 15 cc. of ether, and suction was employed in transferring the solution to the oxidation flask.

Total Fatty Acids—Total fatty acids were determined by the method of Bloor (7).

Total Lipid—Total lipid was calculated as the sum of total fatty acids and total cholesterol.

Residual Fatty Acids—This portion, namely the fatty acids other than those in combination with phospholipid and cholesterol, was obtained by subtracting the sum of the fatty acids present in the phospholipids and in the cholesterol esters from the total fatty acids: total fatty acids − ((0.67 × weight of phospholipid) + (0.73 × weight of esterified cholesterol)). The fatty acid in combination with the cholesterol is assumed to be oleic acid (8).

All solvents employed in this investigation were freshly distilled. Prior to distillation, the alcohol was purified by refluxing over potassium hydroxide for 4 to 6 hours. The ethyl ether was peroxide-free. In the case of petroleum ether, purification was effected before distillation by shaking at intervals with concentrated sulfuric acid over a period of 24 hours.

The determinations of the blood lipids were carried out in triplicate, and the values recorded are the averages of closely agreeing results. Blood sugar was determined with the copper-iodometric reagent of Shaffer and Somogyi (9), the filtrate being obtained by the precipitation of blood with zinc hydroxide (10).

Results

Normal Dogs—The lipids of the whole blood of ten normal dogs are shown in Table I. The total lipid content varied from 644 to 465 mg. per 100 cc. of blood, while the maximum and minimum values for total fatty acids were respectively 439 and 309 mg. The phospholipid values obtained were 391 mg. per 100 cc. of whole blood for the highest and 286 mg. for the lowest. Total cholesterol fluctuated between 137 and 205 mg. per 100 cc. of blood; the free or uncombined portion of this consisted of 144 mg. in the case of the maximum value and 104 mg. in the case of the minimum value, whereas the esterified portion varied from 66 to 29 mg. per 100 cc. of blood, that is from 33 to 19 per cent of the total cholesterol.

The animals may be divided into two groups with regard to the time during which they were kept on the relatively low fat diet. Thus dogs N.A. and N.B. were fed this diet for 2.3 and 1.3 years respectively, while the other normal dogs received it for periods varying from 32 to 102 days. It is interesting to note that the blood lipids of the animals maintained for the longer period on the diet were not lower than those obtained from animals receiving the diet for shorter periods.

TABLE I

Whole Blood Lipids (Postabsorptive) in Normal Dogs

Dog	Sex	Weight	Days on diet	Cholesterol				Total fatty acids	Phospholipids	Residual fatty acids	Total lipid
				Total	Free	Ester					
		kg.		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent of total*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
N. A.	F.	25.0	742	163	123	40	25	399			562
			838	205	144	61	30	439	343	164	644
N. B.	M.	9.2	415	183	126	57	31	423			606
			462	197	131	66	33	434	300	185	631
N. C.	F.	13.0	32	179	142	37	21	398			577
			66	168	135	33	20	402	365	134	570
N. D.*	"	22.0	102	157	124	33	21	385	286	169	542
N. F.	M.	16.8	87	156	127	29	19	309	303	85	465
N. H.	F.	9.3	53	172	132	40	23	425	288	203	597
N. I.	"	12.0	60	163	132	31	19	364	322	125	527
N. J.	"	10.3	93	155	125	30	19	390	391	106	545
N. K.	"	12.5	76	170	136	34	20	382	376	105	552
N. L.	M.	8.8	32	137	104	33	24	370			507

* Laparotomy performed 1 month before animal was put on diet.

Completely Depancreatized Dogs—The blood lipids of ten depancreatized dogs are shown in Table II, and these values, along with those obtained in the normal animals, are summarized in Table III. The results taken as a whole show a marked reduction in the postabsorptive level of the total lipid, total cholesterol, phospholipid, and total fatty acids in the blood of depancreatized animals. In the case of the above lipid constituents, the maximum values obtained in the depancreatized animals were below the mean values found in the normal dogs. The residual fatty acids show a slight decrease in the depancreatized animal as compared with the normal, but it is doubtful whether too much significance should be attached to this constituent, since it is obtained by a calculation of three other experimentally determined values and consequently, as pointed out by Bloor (8), is affected by the errors inherent in each determination.

TABLE II

Whole Blood Lipids (Postabsorptive) in Completely Depancreatized Dogs

Dog	Sex	Weight	Date of pancreatectomy	Period depancreatized when blood sample taken	Cholesterol				Total fatty acids	Phospholipid	Residual fatty acids	Total lipid	Blood sugar
					Total	Free	Ester						
		kg.		*wks.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent of total*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
D. A.*	F.	9.7	Mar. 11, 1931	131	100	97	3	3	286			386	
				141	104	109	−5	−5	262			366	384
D. B.*	M.	8.0	July 27, 1932	59	101	102	−1	−1	226			327	
				72	108†	109	−1	−1	304			412	259
D. C.*	F.	10.7	Sept. 1, 1931	106	102	95	7	7	274			376	
				119	99†	100	−1	−1	216	146	118	315	272
D. D.*	M.	7.8	Sept. 1, 1932	55	114	109	5	4	315			429	
				64	123	120	3	2	288	254	116	411	358
D. E.‡	F.	7.5	Sept. 5, 1932	55	101	100	1	1	260			361	
				64	96	99	−3	−3	236	152	134	332	417
D. F.*	"	9.4	Mar. 2, 1931	133	113	116	−3	−3	269			382	
				140	124	127	−3	−2	347	263	171	471	476
D. G.‡	"	6.7	Aug. 1, 1932	60	112	114	−2	−2	225			337	
				71	108	109	−1	−1	253	223	104	361	304
D. H.§	"	9.7	Sept. 2, 1933	3	116	119	−3	−3	254			370	
				10	116	117	−1	−1	230	207	91	346	246
D. I.§	"	5.5	July 30, 1933	26	111	109	2	2	272	158	165	383	426
D. J.*	"	7.2	June 29, 1932	75	106	106	0	0		228			

* Cataracts were present in animal.
† Blood samples taken 9 hours after previous meal.
‡ Cataracts were absent in animal.
§ Animal was not studied with regard to cataractous changes (1).

The most striking change observed was the decrease in the cholesterol of the blood of the depancreatized animals. The reduction, however, did not occur to the same extent in the different cholesterol fractions. The decrease was most marked in the esterified portion that had completely disappeared from the blood of eight of the ten depancreatized dogs recorded in Table II. In the two remaining animals, namely dogs D.D. and D.I., values ranging from 2 to 4 mg. per cent were found as compared with a range of of 66 to 29 mg. in the normal dogs.

TABLE III

Summary of Blood Lipids of Normal and Completely Depancreatized Dogs

The values are expressed in mg. per 100 cc.

	Normal			Depancreatized		
	Maximum	Minimum	Mean	Maximum	Minimum	Mean
Cholesterol						
Total	205	137	170	124	96	109
Free	144	104	129	127	95	109
Ester	66	29	40	7	0*	1
" as per cent of total	33	19	23	7	0	1
Total fatty acids	439	309	394	347	216	266
Phospholipid	391	286	330	263	146	204
Residual fatty acids	203	85	142	171	91	128
Total lipid	644	465	563	471	315	374
Ratios						
Total fatty acids to total lipid	0.73	0.67	0.70	0.74	0.67	0.71
Total cholesterol to total lipid	0.33	0.27	0.30	0.33	0.26	0.29
Phospholipid to total cholesterol	2.5	1.5	1.9	2.2	1.5	1.9

* Negative values considered as 0.

In the absence of esterified cholesterol, both free and total cholesterol are equal, but inasmuch as the determination of total cholesterol involves one manipulation more than the free, namely saponification, it is to be expected that total cholesterol in this special case would yield results slightly lower than free cholesterol. This no doubt accounts for the negative values for esterified cholesterol, which nevertheless were within the limits of experimental error.

The lipid ratios, namely total fatty acids to total lipid, total cholesterol to total lipid, and phospholipid to total cholesterol, for both normal and depancreatized dogs are shown in Table III. Despite the extensive changes that occurred in the different blood lipid constituents of the depancreatized animals, the mean values of these ratios for the depancreatized dogs were in close agreement with those calculated for the normals. Thus in both depancreatized and normal dogs approximately 70 per cent of the total lipid in the blood was present as fatty acids, a value which agrees closely with the value observed by us in the whole blood lipids of man (11). Moreover, the maximum and minimum deviations from the mean values obtained for the above three ratios were practically the same in normal and depancreatized dogs. Apparently in the presence of the profound disturbance of the blood lipids that had occurred in the depancreatized dog, the normal relation of total fatty acids, total cholesterol, and phospholipid to each other has been maintained.

DISCUSSION

The significant change observed in the blood lipids in the post-absorptive state of the completely depancreatized dogs maintained in good nutritional condition for long periods of time by diet and insulin was a lowering of the lipid level that involved total fatty acids, phospholipid, and cholesterol. In the case of cholesterol a complete disappearance of the esterified portion occurred in most of the animals. These changes in the blood lipids may make their appearance in a relatively short time, for the complete absence of esterified cholesterol from a single dog (D.H.) was observed 3 weeks following pancreatectomy.

In seeking an explanation for the lowered lipid level, and in particular for the absence of esterified cholesterol in the blood of the completely depancreatized dog, it is necessary to consider the following factors: the dietary conditions under which these animals were maintained; the absorption of fats from the intestinal tract in the absence of pancreatic juice; the state of the liver, since it is intimately concerned with lipid metabolism; and finally the degree of diabetes present in these animals. The significance of these factors will be discussed in the above order.

Low Fat Diet—The diet employed in this study is relatively low in fat, but this in itself cannot have produced marked variations in the esterified portion of cholesterol, for Bloor (8) found that decreasing the fat content from approximately 33 to 6 per cent of the caloric value of the diet produced only a small change in the esterified cholesterol of the plasma of dogs. In the present study the blood of the normal dogs that received the same diet as that of the depancreatized animals contained esterified cholesterol in amounts varying from 19 to 33 per cent of the total cholesterol, and it is important to note that animals receiving this diet for as long as 2.3 years, a period comparable with the period of survival of some of the depancreatized dogs, did not show lower esterified cholesterol values than the normal animals receiving this diet for periods as short as 1 month. The foregoing considerations therefore rule out the diet *per se* as a factor instrumental in the disappearance of esterified cholesterol from the blood of the depancreatized dog.

Digestion and Absorption of Fat—The complete absence of pancreatic juice from the intestine of these dogs definitely interferes with the digestion and absorption of fats. Since esterified cholesterol of the blood has been shown to increase during the absorption of fat (12–14), it is quite conceivable that an impairment in this process might lead to a diminished content of combined cholesterol in the blood. The effect of faulty fat absorption upon blood cholesterol has recently been reinvestigated by Hawkins and Wright (15), who produced this by total exclusion of bile from the intestinal tract. These workers found high plasma cholesterol values with a normal ratio of ester to total cholesterol in a dog that had received no bile in its intestine for a period of 7 months following total obstruction of the bile duct. Since the normal cholesterol ester ratio is maintained despite the profound disturbance in the fat assimilation produced by the total absence of bile from the intestine, it seems unlikely that defective digestion and absorption of fats is the causative factor for the disappearance of esterified cholesterol from the blood of the completely depancreatized dog maintained with insulin. Further evidence in accord with this view is supplied by the observations of Ling (14) on the blood lipids of dogs receiving a fat-free diet for a period of 6 months. Although a lowering in the blood lipids was observed, this was due largely to constituents other than cholesterol and lecithin. The diet consisted of 10 parts of dried beef (extracted, fat-free), corn starch 81 parts, yeast 5, and a salt mixture[1] 4 parts. At the most it contained 0.5 per cent fat.

Liver and Bile—The relation of the liver to these blood lipid changes, particularly cholesterol, in the depancreatized dog is obviously of importance, and a study of the lipids of this organ is in progress. Although the part played by the liver in sterol metabolism is at present poorly understood, it has been demonstrated that large amounts of esterified cholesterol accumulate in the liver during the ingestion of diets rich in free cholesterol (16, 17). Moreover, a decrease or even a complete absence of esterified cholesterol from the blood has been found in parenchymatous degeneration of the liver (15, 18–20). This has been ascribed by some workers to an interference in the enzymatic synthesis of esterified cholesterol, a process regarded as largely dependent upon the intact liver cell (18, 19). Still others have advanced the belief that impaired absorption of cholesterol resulting from interference in the excretion of bile has led to the diminished amount of esterified cholesterol in the blood in hepatic disease (20). This latter explanation is not borne out by the recent observations of Hawkins and Wright already referred to above.

It has been repeatedly shown in this laboratory that the bile ducts remain unobstructed in completely depancreatized dogs that have been kept alive for as long as 2 or 3 years. Moreover, Berg, Zucker, and Robin (21) have found no alteration in gall-bladder function in dogs as the result of pancreatectomy. It may be concluded therefore that the disappearance of esterified cholesterol in the blood of the completely depancreatized dogs maintained in this laboratory cannot be ascribed to an absence of bile in the intestine.

The livers of the depancreatized dogs in the postabsorptive state contained as much as 20 per cent or more fat (22). By histological examination it was found that the liver cells contained no normal cytoplasm, the cells being completely filled with fat.[2] Since chronic liver damage in the dog has been shown to diminish or even lead to a disappearance of ester cholesterol from the blood (15), it is not improbable that impaired liver function associated with the accumulation of fat in this organ may be responsible for the absence of esterified cholesterol from the blood of completely depancreatized dogs maintained with insulin. The relation of the abnormal state of the liver cell to the disturbed blood lipid picture, however, requires more detailed investigation. In some preliminary observations on the liver of these animals, it has been found that as much as 75 per cent of the total cholesterol was in the form of the ester (22). The distribution of esterified cholesterol in this animal is therefore striking; it is entirely absent from the blood and comprises the major portion of the cholesterol in the liver.

---

[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 55 (1918).

[2] The histological examination of this tissue was carried out by Dr. C. L. Connor of the Division of Pathology of the University of California Medical School.

Insulin Insufficiency—Blood lipid studies have been the subject of numerous investigations in diabetic subjects (23–27). In patients receiving insulin treatment, the blood lipid level may be normal, although high and low values are by no means uncommon (23, 24). Thus Joslin (23) reported that 60 per cent of 116 insulin-treated diabetics studied during 1926–27 had plasma cholesterol values ranging from 100 to 230 mg. per cent, with an average value of 186, whereas the average value observed in normal subjects was 230 mg. per cent. As regards esterified cholesterol, Bloor, Buckner, and Gibbs (25) found this constituent increased to the extent of 10 to 15 per cent above normal in diabetic patients, most of whom were under insulin treatment. It is clear therefore that the diabetic patient presents no marked disturbance in lipid metabolism so long as sufficient insulin is available to him. However, an increase in all lipid constituents is of frequent occurrence in conditions of insulin insufficiency such as diabetic acidosis or coma. An increased level of the blood lipids has also been observed by Bloor, Gillette, and James (28) in the insulin insufficiency induced in dogs by excision of nine-tenths of the pancreas. These animals, of course, received no insulin treatment following the operation.

As judged by the blood sugars in the postabsorptive state which ranged from 246 to 476 mg. per cent, and by the presence of glucose in the 24 hour sample of urine, the insulin administered to the completely depancreatized dogs in the present study was not sufficient to restore the carbohydrate metabolism to normal. It is difficult at present to determine the significance of this insulin insufficiency with regard to the blood lipid changes encountered in these animals. Since, however, a decrease of ester cholesterol has not been noted in the blood of diabetic patients receiving no insulin treatment, it may be tentatively assumed that the sterol disturbance observed in the depancreatized dogs maintained with insulin is not due to a deficient insulin supply.

SUMMARY

1. The whole blood lipids of completely depancreatized dogs kept alive for long periods of time by means of insulin were studied in the postabsorptive state.

2. A reduction was found in all lipid constituents, namely total fatty acids, phospholipids, and free and esterified cholesterol.

3. The lowering was most marked in the esterified cholesterol fraction. In most of the animals, this constituent had entirely disappeared from the blood.

4. The significance of these lipid changes in the depancreatized dog maintained with insulin is discussed.

BIBLIOGRAPHY

1. Chaikoff, I. L., and Lachman, G. S., *Proc. Soc. Exp. Biol. and Med.*, **31**, 237 (1933).
2. Evans, H. M., and Lepkovsky, S., *J. Nutrition*, **3**, 353 (1931).
3. Okey, R., *J. Biol. Chem.*, **88**, 367 (1930).
4. Yasuda, M., *J. Biol. Chem.*, **92**, 303 (1931).
5. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
6. Page, I. H., Pasternack, L., and Burt, M. L., *Biochem. Z.*, **223**, 445 (1930).
7. Bloor, W. R., *J. Biol. Chem.*, **77**, 53 (1928).
8. Bloor, W. R., *J. Biol. Chem.*, **95**, 633 (1932).
9. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
10. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
11. Chaikoff, I. L., McGavack, T. H., and Kaplan, A., *J. Clin. Inv.*, **13**, 1 (1934).
12. Knudson, A., *J. Biol. Chem.*, **32**, 337 (1917).
13. Bodansky, M., *Proc. Soc. Exp. Biol. and Med.*, **28**, 628 (1931).
14. Ling, S. M., *Chinese J. Physiol.*, **5**, 381 (1931).
15. Hawkins, W. B., and Wright, A., *J. Exp. Med.*, **59**, 427 (1934).
16. Okey, R., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1003 (1933).
17. Chanutin, A., and Ludewig, S., *J. Biol. Chem.*, **102**, 57 (1933).
18. Epstein, E. Z., *Arch. Int. Med.*, **47**, 82 (1931); **50**, 203 (1932).

19. Thannhauser, S. J., Lehrbuch des Stoffwechsels und der Stoffwechselkrankheiten, Munich, 506 (1929).
20. Gardner, J. A., and Gainsborough, H., *Quart. J. Med.*, **23**, 465 (1930).
21. Berg, B. N., Zucker, T. F., and Robin, P., *Proc. Soc. Exp. Biol. and Med.*, **31**, 106 (1933).
22. Chaikoff, I. L., and Kaplan, A., unpublished data.
23. Joslin, E. P., The treatment of diabetes mellitus, Philadelphia, 4th edition, 229 (1928).
24. White, P., Diabetes in childhood and adolescence, Philadelphia, 79 (1932).
25. Bloor, W. R., Buckner, E., and Gibbs, C. B. F., *Proc. Soc. Exp. Biol. and Med.*, **30**, 63 (1932).
26. Rabinowitch, I. M., *Arch. Int. Med.*, **43**, 363, 372 (1929).
27. Man, E. B., and Peters, J. P., *J. Clin. Inv.*, **13**, 237 (1934).
28. Bloor, W. R., Gillette, E. M., and James, M. S., *J. Biol. Chem.*, **75**, 61 (1927).

THE EFFECT OF THE SATURATED FATTY ACID CONTENT OF THE DIET ON THE COMPOSITION OF THE BODY FAT

BY A. D. BARBOUR

(*From the Department of Biochemistry, Ontario Research Foundation, Toronto, Canada*)

(Received for publication, March 24, 1934)

INTRODUCTION

During the course of an investigation of the fate of ingested isooleic acid in the animal body (1), diets containing large amounts of lard and hydrogenated shortening were fed to groups of albino rats. The diet was as follows:

	per cent
Casein (commercial)	10
Sucrose	5
Salt mixture (Osborne and Mendel)*	4
Sodium chloride	1
Corn-starch	40
Lard or shortening	40
	100

A small amount of fresh spinach was also fed daily. The lard and shortening had approximately the same total unsaturation as measured by the iodine value, but differed in saturated acid content by about 8 per cent. When the body fat from the animals was examined, the average iodine values of the fats from the two groups were equal, but the fats were found to differ only slightly in their content of saturated acids. When the feces from the two groups over the test period were examined, however, very wide differences were found. The fecal fat from the shortening-fed group was not widely different in composition from the dietary fat, while the fecal fat from the lard-fed group contained more than 60 per cent of saturated acids, with a correspondingly low iodine value and high melting point. The average values for the composition of the body and fecal fats are shown in Table I. Apparently, when the dietary fat had a higher saturated acid content than the normal body fat, the saturated acid content of the latter was not raised, as would be expected, but remained approximately constant, while the excess dietary saturated acids were rejected and excreted.

---

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

The general effect of the unsaturation of the dietary fat on the unsaturation of the body fat has been studied frequently (2–5) but there has been no study made of the saturated acids separately. Arnschink (6) noted that the fecal fat of dogs receiving mutton fat showed a higher melting point than the food fat, but did not relate this to the composition of the body fat. Munk and Rosenstein (7) observed that under similar conditions the chyle fat showed a lower melting point than the dietary fat, which might indicate that a portion of the saturated acids in the dietary fat had remained unabsorbed. Somewhat similar results have been reported by Bloor (8), but the body fat was not examined.

TABLE I

Average Analyses of Body and Fecal Fat from Animals Fed Lard and Hydrogenated Shortening on a 40 Per Cent Level

	No. of animals	Iodine value of dietary fat	Saturated acids in dietary fat	Iodine value of body fatty acids	Saturated acids in body fatty acids	Iodine value of fecal fatty acids	Saturated acids in fecal fatty acids
			per cent		*per cent*		*per cent*
Test A							
Lard diet..................	5	63.7	35.7	73.9	27.3	33.4	63.4
Shortening diet...........	4	63.3	27.7	74.0	24.6	67.7	32.8
Test B							
Lard diet..................	10	63.7	35.7	76.6	26.6	23.6	67.1
Shortening diet...........	14	63.3	27.7	76.6	25.6		

It was thought of interest to make a further study of this peculiar behavior of saturated acids, using a variety of dietary fats of varying saturated acid content, and observing both the saturated acid content and the general unsaturation of the resulting body fat. The results of the former experiments (1) were obtained under somewhat abnormal conditions, since the animals were fasted before the test in order to deplete their reserve fat, and were then fed a restricted synthetic diet with a 40 per cent fat level. In the experiments to be described, the rats received a normal adequate diet, to which 20 per cent of the test fat was added. Young growing rats were used, and the feeding was continued over a period of 2 months. Linseed oil, peanut oil, cottonseed oil, and lard were the fats used for test, with saturated acid contents ranging from 7 to 30 per cent. In addition, a control group of rats was kept on the stock diet alone. At the end of the test period, the body and fecal fatty acids were extracted and analyzed. The liver fats were examined separately, and in addition the fatty acids from the peanut oil-fed group were examined for the presence of arachidic acid.

TABLE II

Composition of Fats Used in Test Diets

	Linseed oil	Peanut oil	Cottonseed oil	Lard	Stock diet
Iodine value, glycerides	185.0	98.1	107.4	63.7	66.7
" " mixed fatty acids	193.8	102.7	112.6	66.7	69.9
Solid acid content, *per cent*	7.2	17.0	22.0	36.1	27.4
Iodine value, solid acids	3.0	2.8	0.9	1.0	4.0
Saturated acids, *per cent*	7.0	16.5	21.8	35.7	26.2
Calculated composition of fat in diets as fed					
Fat content of diet, *per cent*	23.16	23.16	23.16	23.16	3.95
Iodine value, fatty acids	177.0	98.3	106.7	67.2	69.9
Saturated acids, *per cent*	9.6	17.8	22.4	34.4	26.2

EXPERIMENTAL

The animals used for the test were selected from the laboratory stock, and were scattered with respect to sex and litter.

The basic diet was a commercial mixed feed, containing mixed grains, barley malt, molasses, meat scrap, buttermilk powder, and cod liver oil. This is used on account of its cheapness and convenience of form, and has proved completely adequate for growth and reproduction over a period of several years.

The fat samples used for the test were authentic samples obtained in Toronto. The analyses of these fats and of the fat in the stock diet are shown in Table II. The fat in the stock diet amounted to 3.95 per cent, with iodine value 66.7 and saturated acid content 26.2 per cent. The actual fat content and composition of the diets *as fed* were calculated from these values and are also shown in Table II.

The animals were placed on the experiments at the age of 28 days, and the feeding continued for 60 days. The average weight records for each group are shown in Table III. It will be noted that better growth was made on the control diet than on any of the high fat diets, the poorest growth being shown by the group receiving cottonseed oil.

TABLE III

Feeding Record Averages and Average Iodine Value and Saturated Acid Content of Body Fat

Diet	No. of animals	Average body weight			Final body weight	Total gain in body weight (average)	Fat content	Iodine value, total mixed fatty acids	Saturated acid content
		At start of test	20 days after start of test	40 days after start of test					
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*		*per cent*
Control...........	7	59	122	207	216	157	10.0 (σ1.8)	88.5 (3.0)	27.2 (0.5)
Linseed oil.........	6	49	80	144	158	109	8.8 (σ1.8)	103.0 (8.8)	16.4 (0.7)
Peanut "	8	71	87	166	184	113	9.8 (σ3.0)	88.1 (5.4)	18.3 (0.8)
Cottonseed oil......	7	47	69	110	123	76	10.6 (σ1.3)	97.7 (4.7)	24.6 (1.2)
Lard..............	7	49	85	151	163	114	10.4 (σ2.0)	75.4 (4.1)	25.7 (0.4)

At the end of the feeding period, the rats were electrocuted and the body fat extracted by the modification of Liebermann's method described in detail in the previous paper (1). The livers were removed and examined separately. Iodine values were determined by the method of Wijs (9) and saturated acids by the lead salt-alcohol method of Twitchell (10) as modified by Baughman and Jamieson (11).

The results are also shown in Table III. In order to economize space, only the average results and standard deviations for each group are given. The standard deviation is calculated from the equation (12)

$$\sigma = \sqrt{\Sigma(d^2)/n}$$

where σ = standard deviation, d = deviation of each individual result from the average, and n = number of results.

TABLE IV

Analysis of Fecal Fatty Acids and Liver Fat

Diet	Feces				Liver			
	Fatty acid content	Iodine value of total mixed fatty acids	Saturated acid content of fat	Saturated acid content of feces	Average weight	Average fat content	Saturated acid content of fat	Iodine value of fat
	per cent		*per cent*	*per cent*	*gm.*	*per cent*	*per cent*	
Control	2.0	58.0	47.9	0.96	7.75	3.13	31.4	149.8
Linseed oil	4.2	79.7	29.6	1.24	6.25	4.11	27.0	136.1
Peanut "	12.6	47.8	57.0	7.18	5.90	5.09		141.0
Cottonseed oil	9.9	59.5	46.4	4.60	4.89	4.96	28.1	109.8
Lard	13.5	44.1	60.3	8.14	6.04	5.02		

It is evident that while the iodine values of the body fats are dependent on the iodine value of the dietary fat, the saturated acid content is dependent on the saturated acid content of the diet. Thus, although peanut oil contains less saturated and more unsaturated acids than cottonseed oil, its iodine value is lower, and the same relationship holds for the body fats of the two groups receiving these oils in the diet.

The feeding of lard, however, does not produce a body fat having a proportionately high saturated acid content. Although the saturated acid content of the lard is 14 per cent greater than that of the cottonseed oil, the body fats of the animals receiving these fats differ by only 1.1 per cent in saturated acid content, and both are slightly lower than the control group on the normal diet.

The feces were collected over the test period, those from the animals in each group were combined, and the fat extraction and analysis carried out in the same way as for the body fat. The results are shown in Table IV.

The fecal fat from the lard-fed animals shows the highest fat content, and also the highest saturated acid content; that from the peanut oil-fed animals is next, for reasons which will appear below. The sample from the linseed oil-fed animals is little higher than that of the control, indicating almost complete utilization of this oil. It is interesting to note that under these conditions of feeding, the saturated acid content is in all cases higher in the fecal fat than in the dietary fat, indicating that a portion of the saturated acids is unabsorbed in all cases, even with linseed oil.

It has been shown by Best and his collaborators (13, 14) that rats fed diets containing high proportions of fat, particularly saturated fat, have a tendency to develop abnormally fatty livers. To ascertain whether liver damage had taken place during our test, the livers were removed and the total fat content separately estimated. (These estimations were made by the kindness of Miss J. H. Ridout of the Department of Physiological Hygiene, University of Toronto.) The samples of liver fat from each group were combined for the determination of saturated acids and iodine value. The sample from the group fed lard was lost by accident, and there was an insufficient amount of the sample from the peanut oil-fed group for a determination of saturated acids. The average results are shown in Table IV. There is no evidence of fatty livers; the iodine values are high, as would be expected, but there is no evidence of any proportionality between the composition of the liver fat and the dietary fat.

It has been stated by Gill and Vaala (15) that the lard from hogs which have been fed peanuts does not contain arachidic acid. It was thought of interest to determine whether or not the body fat of the rats which had received peanut oil in these tests showed the presence of this acid. 5 gm. composite samples of the body fatty acids from this group were examined for arachidic acid by the method of Evers (16). About 5 mg. of crude precipitate were obtained, which on recrystallization from 70 per cent alcohol gave insufficient material for a melting point determination. Even if all of this precipitate is arachidic acid, the amount in the body fat does not exceed 0.1 per cent. The fecal fat was also examined for the presence of arachidic acid. A 3.07 gm. sample of the mixed fecal fatty acids gave 0.736 gm. of crude arachidic acid, which on recrystallization from 90 per cent alcohol melted at 71.9°. This acid thus makes up at least 24 per cent of the fecal fatty acids from this group, and accounts in large measure for the high fecal fat concentration and high saturated acid content.

DISCUSSION

It would appear from these results that there is a maximum level or threshold for saturated acids in the body fat of the albino rat, occurring at a saturated acid content of about 25 to 27 per cent. The feeding of fats with low saturated acid content may lower this level, but the feeding of a fat with higher saturated acid content does not raise it. This behavior of the saturated acids cannot be accounted for by the melting point of the fats, and bears no relation to the iodine value.

Since the saturated acid content of the fecal fat is in all cases higher than that in the dietary fat, it would also seem possible that there may be a threshold for each individual fatty acid. This view would seem to be supported by the fact that arachidic acid is excreted almost quantitatively, only traces being deposited in the body fat. It would be of interest to study the feeding of synthetic mixtures containing only one saturated acid, in different concentrations, in order to determine whether such thresholds exist for other fatty acids.

SUMMARY

1. In the albino rat fed fat at a 20 per cent level, the saturated acid content of the body fat is proportional to the saturated acid content of the diet, while the unsaturation of the body fat, as measured by the iodine value, is proportional to the unsaturation of the dietary fat.

2. The saturated acid content of the body fat may be lowered by feeding fats of low saturated acid content, but cannot be raised beyond a level of 25 to 27 per cent by feeding fats of higher saturated acid content. In the latter case, the excess saturated acid is excreted in the feces, which may thus have very high saturated acid

contents. The existence of a threshold for saturated acids in the body fat is thus indicated.

3. Linseed oil, peanut oil, cottonseed oil, and lard, when fed to albino rats on a level of 20 per cent in a diet otherwise normal, do not cause abnormal deposition of fat in the liver.

4. The arachidic acid of peanut oil is almost quantitatively excreted by the albino rat. If present in the body of peanut oil-fed rats, its concentration does not exceed 0.1 per cent.

The author wishes to express his thanks to Dr. H. B. Speakman, Director of the Foundation, for his interest and advice, and to Mr. Arthur Hurndall for his assistance in the analytical work and care of the animals.

BIBLIOGRAPHY

1. Barbour, A. D., *J. Biol. Chem.*, **101**, 63 (1933).
2. Lebedev, A., *Centr. med. Wissensch.*, No. 8 (1882); *Arch. ges. Physiol.*, **31**, 11 (1883).
3. Henriques, V., and Hansen, C., *Skand. Arch. Physiol.*, **11**, 151 (1901).
4. Hankins, O. G., and Ellis, N. R., *U. S. Dept. Agric.*, *Bull. 1407* (1926).
5. Hankins, O. G., Ellis, N. R., and Zeller, J. H., *U. S. Dept. Agric.*, *Bull. 1492* (1928).
6. Arnschink, L., *Z. Biol.*, **26**, 434 (1890).
7. Munk, I., and Rosenstein, A., *Arch. path. Anat. u. Physiol.*, **123**, 230, 484 (1891).
8. Bloor, W. R., *J. Biol. Chem.*, **11**, 429 (1912); **15**, 105 (1913); **16**, 517 (1913–14).
9. Wijs, J. J. A., *Chim. et ind.*, **20**, 1043 (1928).
10. Twitchell, E., *J. Ind. and Eng. Chem.*, **13**, 806 (1921).
11. Baughman, W. F., and Jamieson, G. S., *Oil and Fat Ind.*, **7**, 330 (1930).
12. American society for testing materials; manual for interpretation of refractory test data, Philadelphia, 2nd edition (1931).
13. Best, C. H., Hershey, J. M., and Huntsman, M. E., *J. Physiol.*, **75**, 56 (1932).
14. Best, C. H., and Huntsman, M. E., *J. Physiol.*, **75**, 405 (1932).
15. Gill, A. H., and Vaala, G. T., *Science*, **74**, 548 (1931).
16. Evers, N., *Analyst*, **37**, 487 (1912).

PEPTIC HYDROLYSIS OF INSULIN

BY A. M. FISHER AND D. A. SCOTT

(*From the Connaught Laboratories, University of Toronto, Toronto, Canada*)

(Received for publication, June 1, 1934)

Investigations dealing with the chemistry of insulin show that this substance bears a striking resemblance to proteins. A few of the characteristic properties might be enumerated. Crystalline preparations of insulin have an isoelectric point at which the material is insoluble. The elemental composition of the crystalline material is similar to that of proteins. It has been shown that these crystals contain the amino acids, cystine, tyrosine, arginine, histidine, lysine, leucine, and glutamic acid (1). Tryptophane (2) and thiomethyl (3) (the latter originating from methionine) have not been detected. Moreover, the theory that insulin is associated with a protein is supported by the fact that the physiological activity of the hormone is destroyed by proteolytic enzymes. Dirscherl (4) has shown that the destruction of insulin by pepsin is a true example of peptic hydrolysis.

With this knowledge of the chemical nature of insulin, the question of the association of the physiological activity with the complete molecule, or with some group or groups (say some amino acid), becomes of interest. It has been suggested, in view of experiments with acidic ethyl alcohol, that a carboxyl group may be concerned. Freudenberg has found evidence of two amino groups, differing in some respects, which are also essential (5). It has been reported that the free amino groups in insulin can be removed by the Van Slyke procedure, without completely destroying the potency (5, 6). Thus, the physiological action of the hormone cannot be due solely to some definite arrangement of amino groups. From measurements of the absorption bands produced by insulin, Kuhn, Eyer, and Freudenberg (7) have suggested that tyrosine and cystine are of importance when one is considering the constitution of insulin. They have found it possible, however, to inactivate insulin with acid or alkali without effecting any change in the absorption spectra. Thus it can be said that inactivation may proceed, under certain conditions, without destruction of tyrosine. Furthermore, these authors believe that the physiologically active group in insulin may contain an asymmetric carbon atom. The problem has also been studied by a consideration of zymatic hydrolysis of insulin. Charles and Scott (8) have found that in the hydrolysis of crystalline insulin with pepsin or trypsin the destruction of the physiological activity progresses at a much greater rate than does the hydrolysis of the protein. This work was confirmed by Freudenberg *et al.* (9), who also reported that acetyl insulin digested with pepsin or trypsin could not be reactivated.

As noted above, in peptic digestion of insulin the destruction of the physiological activity proceeds at a much greater rate than does the hydrolysis of the protein. This fact might be explained in two ways. It may be possible that the intact protein is essential for the physiological activity. During hydrolysis, the protein would be decomposed into two or more inactive fractions. On the other hand, the rapid loss of potency may be due to the cleavage of some peptide or amino acid responsible for the hypoglycemic properties. In an attempt to shed further light on this problem, it was planned to hydrolyze crystalline insulin slowly with crystalline pepsin and to remove samples of the digest at various times, correlating the potency with the composition of the undigested protein. In this way it was thought that if the loss in potency during hydrolysis was due to any particular constituent of the insulin protein, it might be detected in the chemical analysis.

EXPERIMENTAL

In view of the fact that only a limited quantity of crystalline insulin was available for this work, it was necessary to become familiar with a process which would permit the analysis of a small quantity of material, *viz.* about 200 mg. After attempting the analysis of 200 mg. samples of gelatin by several methods, it was found that consistent results for the nitrogen fractions enumerated in Van Slyke's scheme could be obtained by using a modified form of Hanke and Koessler's (10) procedure for the precipitation of the basic amino acids in protein hydrolysates. (This process is described in detail later in the present paper.) Samples of amorphous insulin (15 international units per mg.) were then analyzed by this method. The results which were obtained with three samples of this material are recorded in Table I. Since the results agreed in general, it was decided to repeat the procedure with 200 mg. of crystalline insulin (Sample A) and to compare the results with those reported by other investigators who used larger samples of insulin. The results of this experiment together with the values obtained by other workers are recorded in Table II. Although certain values show considerable variation, the results obtained in this work are in general agreement with those of other investigators. Accordingly, the use of this method seemed justifiable. Since some interest has been attached to the difference in cystine values, depending upon whether the method of Folin and Marenzi (11) or Sullivan's (12) procedure was used, it was decided to plan for the use of a larger sample (225 mg.) and determine the cystine by both methods.

TABLE I

Nitrogen Distribution in Amorphous Insulin

200 mg. of amorphous insulin were used; all values are in per cent of total nitrogen.

	Sample A	Sample B	Sample C
Ammonia N	8.4	9.7	8.0
Humin N	0.07	0.1	0.13
Total “ monoamino fraction*	63.0	62.3	63.3
Amino “ “ “ *	64.5	64.0	63.3
Histidine N	6.7	5.75	5.15
Cystine N (Folin)	8.7	8.0	8.3
Arginine N	8.95	8.8	13.5
Lysine N	5.3	6.5	3.5
Distillate from removal of HCl	0.09	0.3	0.18
Total	101.2	101.4	102.1

* Calculated on a cystine-free basis.

The crystalline insulin used in this research had been recrystallized from a phosphate buffer solution and contained 25 international units per mg. The pepsin used was also crystalline and was prepared for us by Dr. John Northrop. A preliminary experiment with 100 mg. of crystalline insulin was conducted to determine the quantity of pepsin to be used and the times at which samples should be removed to assure a reasonable difference in potency. In this experiment, samples of the digest were removed every 10 minutes. After a few biological assays, it seemed evident that a suitable difference in potency in the three samples which we proposed to remove could be obtained if the peptic digestion was conducted as described below.

TABLE II

Nitrogen Distribution in Insulin

Figures are in per cent of total nitrogen.

	Dickens, Dodds, Lawson, and Maclagan*	Wintersteiner, du Vigneaud, and Jensen†	Present investigation
Ammonia N	9.59	9.58	10.45
Humin N	0.52	2.42	0.14
Cystine N	5.93	6.08	9.56
Histidine N	4.29	7.60	5.36
Arginine N	9.37	6.60	11.0
Lysine N	0.21	2.76	2.1
Monoamino fraction, total N	72.75	64.61	63.7‡
" " amino "	67.61	56.30	61.5‡
Total	102.6	99.6	102.3

* Dickens, F., Dodds, E. C., Lawson, W., and Maclagan, N. F., *Biochem. J.*, **21**, 560 (1927).

† Wintersteiner, O., du Vigneaud, V., and Jensen, H., *J. Pharmacol. and Exp. Therap.*, **32**, 397 (1928).

‡ Calculated on a cystine-free basis.

Peptic Digestion—2.700 gm. of crystalline insulin were dissolved in a dilute aqueous solution of hydrochloric acid. The reaction of the solution was adjusted by the addition of N hydrochloric acid until the acidity was pH 1.8. The volume of the solution was 190 cc. As a control sample, 0.50 cc. was removed (Sample A-1) and placed in a test-tube. Both solutions were then put in a water bath at 38°. The flask and the test-tube were covered to avoid any change in concentration of the solutions due to evaporation.

After 15 minutes, 8.1 cc. of a solution containing 0.10 mg. of crystalline pepsin per cc. were added to the solution in the digestion flask. The acidity of the digest was then pH 1.53. In 20 minutes, 0.50 cc. of the digest was removed and added to 9.50 cc. of stock ammoniacal isotonic saline. (The stock solution contained sufficient ammonia to assure the sample for biological assay being alkaline after the addition of the acid sample from the digestion flask. Further destruction of potency of the insulin by peptic hydrolysis was thus prevented.) This solution was kept at 8° and used later for biological assay. Immediately after removing the 0.50 cc. sample, 40.0 cc. of the digest were removed and the protein (Sample B) was precipitated by the addition of 20.0 cc. of 10 per cent trichloroacetic acid. (Previous experiments have shown that this concentration is necessary for the quantitative precipitation of insulin.) After standing for 1 hour, the mixture was centrifuged and the precipitated protein washed and dried as described later. ½ hour after the removal of the first sample, or 50 minutes after adding the pepsin, two samples were removed, *viz.* Sample C-1, 1.00 cc., and Sample C, 60.0 cc. The smaller sample was added to 9.0 cc. of the stock ammoniacal isotonic saline and placed in the refrigerator. The protein in the larger sample was precipitated by the addition of 30.0 cc. of 10 per cent trichloroacetic acid. 40 minutes later, that is, 1½ hours after the digestion commenced, 10.0 cc. of the digest were removed (Sample D-1) and added to 15.0 cc. of ammoniacal isotonic saline. The protein (Sample D) in 80.0 cc. of the digest was precipitated by the addition of 40.0 cc. of 10 per cent trichloroacetic acid. The acidity of the remainder of the digest at the end of the experiment was pH 1.62. 15 minutes after the conclusion of the digestion the control sample mentioned above was removed from the bath and diluted to 89.0 cc. with ammoniacal isotonic saline. This solution, together with all others to be used for biological assay, was placed in the refrigerator until required.

Treatment of Protein Precipitates—The protein in each of Samples B, C, and D was precipitated by the addition of trichloroacetic acid in the manner already described. After standing for 1 hour at 20°, the mixtures were centrifuged. The nitrogen in the supernatant liquid was determined by the micro-Kjeldahl method of Pregl. From this value, the extent of hydrolysis was calculated.

The precipitate was shaken with 40 or 50 cc. of absolute ethyl alcohol until nearly all the protein had dissolved. It was then reprecipitated by the addition of 175 to 200 cc. of ether and placed in the refrigerator. After 14 hours the mixtures were centrifuged and washed with 50 cc. of ether. The samples were again centrifuged and then dried *in vacuo*. From the preliminary experiments it was found that this treatment removed the trichloroacetic acid, which interferes with the determination of tyrosine. Samples of each of these three precipitates were removed for biological assay and estimation of nitrogen distribution as described below.

Analysis of Samples—The procedure used to determine the amount of the various amino acids contained in the fractions removed from the digestion flask was one developed from modifications of the Van Slyke method (13) for the determination of nitrogen distribution. For the analysis of each of Samples A, B, C, and D 325 mg. of material were required. Of this, 100 mg. were used for duplicate determinations of tyrosine, and the remainder (225 mg.) for the estimation of the other values reported in Table III.

Tyrosine was determined by the method of Folin and Marenzi (14), with duplicate samples of 50 mg. each. The material was hydrolyzed with 20 per cent sodium hydroxide at 100° for 14 hours. Since the hydrolysates were practically colorless, the treatment with kaolin, as described in their paper, was omitted.

The 225 mg. sample was used for the determination of cystine, histidine, arginine, lysine, and for the other forms of nitrogen listed in Van Slyke's scheme of nitrogen distribution. In the present investigation, the sample was hydrolyzed according to the method of Rosedale and da Silva (15). We have found it convenient to use a cylindrical Pyrex flask (about 3 × 20 cm.) equipped with a condenser fitted to the flask by a ground glass joint. The sample was placed in the flask, 10.0 cc. of 25 per cent HCl were added, and the mixture gently heated. After all the material had dissolved, the hydrolysis was continued in a sulfuric acid bath for 35 hours at 135–140°. At the end of this time, the hydrolysate was poured into a 30.0 cc. volumetric flask and the Pyrex flask washed with distilled water. The washings were added to the hydrolysate and the flask was filled to the mark with distilled water. Samples were removed from this solution for the determination of cystine.

The *cystine* content was determined colorimetrically by the method of Folin and Marenzi (11) and also by that of Sullivan (12). For analysis by the first method, two 2.0 cc. samples were pipetted into large test-tubes. A series of tubes, each containing a different amount (1.6 to 2.4 cc.) of a standard cystine solution, was also arranged. This standard solution was prepared by dissolving 100.0 mg. of cystine in 100.0 cc. of 1 N hydrochloric acid. To each tube, 2.0 cc. of 20 per cent sodium sulfite were added and the solutions allowed to stand for 1 minute. In preliminary experiments, it was found that the acidity of the solutions was a factor in the degree of color produced. Tompsett (16) has suggested the use of sodium bicarbonate as a neutralizing agent. It was our experience that consistent results were obtained only when a saturated solution of sodium bicarbonate was added to all the tubes until effervescence ceased. Care was taken to add the sodium bicarbonate slowly in order to avoid loss of the solution by splashing during the evolution of carbon dioxide. The determination was then continued as outlined by Tompsett, the improved uric acid reagent described by Folin and Marenzi (17) being used. The unknown solutions were always compared with at least two standards. For the determination of cystine by the method of Sullivan, two 1.0 cc. samples of the hydrolysate were pipetted into test-tubes. Another series of tubes containing varying amounts (3, 4, 5, and 6 cc.) of a standard cystine solution (20 mg. of cystine per 100 cc. of 0.1 N HCl) was prepared. The volume in each tube was adjusted to approximately 6 cc. with distilled water and sodium hydroxide was added until the solutions were neutral to litmus. The volume in each tube was diluted to 10.0 cc. with distilled water. The rest of the determination was as described by Sullivan.

The estimation of cystine completed, the analysis of the hydrolysate was continued according to the method of Hanke and Koessler, with modifications to make the method suitable for the small amount of material which we were using. The hydrolysate (now a volume of 24.0 cc.) together with about 25 cc. of distilled water was poured into a 200 cc. Claisen flask. The hydrogen chloride was removed from this solution by distillation at 45° under reduced pressure. When the volume in the Claisen flask was reduced to about 2 cc., 50 cc. of distilled water were added and the distillation resumed until the volume was again about 2 cc. This process was repeated twice. The last time distillation was continued until only about 1 cc. remained in the Claisen flask. A small quantity of nitrogen could be detected in the distillate. This was determined by the micro-Kjeldahl method of Pregl. The flask and contents were then dried at 80° for 2 hours to remove the last traces of HCl. At the end of this time, 50 cc. of distilled water were added to the residue in the flask and the solution was made alkaline to litmus with a small quantity of calcium oxide. The ammonia was removed by distillation at 45° under reduced pressure for about 25 minutes. The quantity of ammonia was determined by collecting it in two bulbs, immersed in ice, each bulb containing 20.00 cc. of 0.0100 N sulfuric acid. The material remaining in the flask after distillation was filtered through a small Buchner funnel. The flask and residue were washed with hot distilled water until the washings failed to give a positive test with the Pauly reaction. The nitrogen in the residue was determined and recorded as "humin nitrogen." The filtrate and washings were transferred to an evaporating dish, acidified with a small quantity of N hydrochloric acid, and evaporated to dryness on a steam bath. This residue was dissolved in 10.0 cc. of an aqueous solution containing 1.8 cc. of 37 per cent hydrochloric acid and transferred to a 50 cc. centrifuge tube. 1.5 gm. of phosphotungstic acid, which had been carefully purified according to the method of Winterstein (18), were dissolved in 10.0 cc. of water. After heating these two solutions to about 90° in a water bath, the phosphotungstic acid solution was poured into the centrifuge tube containing the hydrolysate. The heating was continued for ½ hour. As the solution cooled slowly to room temperature, the basic amino acids, together with some cystine, precipitated. The mixture was placed in a refrigerator for 48 hours, and then packed in ice for 24 hours. A solution saturated with respect to arginine and histidine and containing 0.9 cc. of 37 per cent hydrochloric acid and 7.5 gm. of phosphotungstic acid per 100 cc. was similarly cooled. Both mixtures were centrifuged. The supernatant liquid from the tube containing the solution for analysis was poured into a 50 cc. volumetric flask. The precipitated basic amino acids were cooled to 0° and washed with 10 cc. of the supernatant liquid from the phosphotungstate solution. After centrifuging, the supernatant liquid was poured into the volumetric flask and the washing repeated with another 10 cc. of the phosphotungstate solution. The mixture was centrifuged again and the supernatant liquid added to the solution of the monamino acids in the volumetric flask. This was then diluted with water to 50.0 cc. The total nitrogen in the monoamino fraction was determined by removing two 2.0 cc. samples from the flasks. The estimations were conducted according to the micro-Kjeldahl method of Pregl. Similar samples were removed to determine the nitrogen contained in the primary amino groups. For this purpose, a micro-Van Slyke apparatus was used.

The basic amino acids (the precipitate in the centrifuge tube) were dissolved in 3 cc. of 1 N sodium hydroxide and diluted to 15.0 cc. with distilled water. Immediately after dilution, two 2.0 cc. samples were removed and acidified with 1 or 2 cc. of 1 N hydrochloric acid. These samples were used to determine the quantity of cystine which had been precipitated in the basic fraction. The procedure used was the modified Folin and Marenzi method already described. For the determination of histidine, 1.0 cc. was removed from the volumetric flask and diluted with 3 or 4 cc. of distilled water. It was then made neutral to litmus with 0.01 N hydrochloric acid and diluted to 10.0 cc. with distilled water. 1.0 cc. samples of this were treated with the reagents described by Hanke and Koessler and compared with their CR-MO indicator (10). The indicator was prepared fresh daily and standardized by comparison with a solution of histidine monohydrochloride. From the original dilution of the basic fraction, two 2.0 cc. samples were removed for the determination of the nitrogen in the primary amino groups and two 1.0 cc. samples for the determination of the total nitrogen.

From the determinations made above, knowing that insulin contains no tryptophane, the values listed in Table III were calculated. Corrections for the solubility of histidine and arginine have been made, the values determined by Van Slyke and by Hanke and Koessler being used.

The sulfur content of Samples B, C, and D was determined, with the following results. Sample B, 3.6 per cent; Sample C, 3.4 per cent; Sample D, 3.6 per cent. If it is supposed that all the sulfur is present as cystine, the cystine-nitrogen values expressed as per cent of the total nitrogen are as follows: Sample B, 11.1 per cent; Sample C, 10.4 per cent; Sample D, 11.2 per cent.

Attempts were made to crystallize Samples B, C, and D. When the experiments were conducted at the usual acidity, pH 6.2, amorphous products were obtained. When, however, the reaction of the solution was adjusted to pH 6.8, needle-like crystals were obtained from each sample. These crystals were removed by

TABLE III

Nitrogen Distribution in Samples of Various Potencies

Figures are in per cent of total nitrogen.

Sample N, *per cent* Potency, *units per mg*	A 14.2 25	B 14.2 19.8	C 14.3 14.8	D 14.1 10.7
Ammonia N	10.45	10.05	10.44	10.84
Humin N	0.14	0.06	Very small	0.12
Cystine N (Folin)	9.56	9.34	10.3	9.9
" " (Sullivan)		10.9	12.4	12.5
Histidine N	5.36	6.75	6.75	6.99
Arginine N	11.0	9.64	12.1	12.5
Lysine N	2.1	3.4	4.85	6.1
Tyrosine N	5.93	5.55	4.76	4.39
Monoamino fraction, total N*	63.7	54.4	55.7	53.4
" " amino "*	61.5	53.6	54.2	53.5
Distillate from removal of HCl	0.65	3.72	3.67	5.23
Total	103.0	97.4	104.8	105.1

* Calculated on a cystine-free basis.

TABLE IV

Biological Assays

Original material contained 25 international units per mg.

Sample	Time after adding pepsin	Protein hydrolyzed	Potency destroyed*	Units of insulin per mg. in pptd. protein
	min.	*per cent*	*per cent*	
Control†	105	0.0	0	26
B	20	6.3	13	19.8
C	50	11.0	57	14.8
D	90	12.0	77	10.7

* Determined from samples of the digest (Samples A, B-1, C-1, and D-1).

† No pepsin was added to this sample.

centrifugation, washed with water, and dried *in vacuo.* Biological assays showed that they contained about 5 international units of insulin per mg. When, however, similar experiments were conducted with control tubes containing insulin which had been crystallized by the phosphate buffer method, no crystals were formed at pH 6.8.

Biological Assays—The physiological activity of the various samples described in this paper has been determined by the mouse method of assay. The values have been obtained by comparison of the unknown sample with a standard insulin solution. At least 200 mice were used for the assay of each sample.

The results of the assays of the three samples (Samples B-1, C-1, and D-1) which were removed from the flask at definite intervals during the digestion are reported in Table IV. For comparison, the physiological activity of the protein which was precipitated in each sample by the addition of trichloroacetic acid is also listed, together with the value determined for the control sample which was removed from the flask before adding the pepsin. This sample was subject to the same conditions of heat and acidity as the larger volume of solution.

DISCUSSION

A method for determining the nitrogen distribution in small quantities of proteins has been described. The procedure was first applied to an amorphous preparation of insulin. Since reasonably consistent results were obtained (Table I) the method was applied to a small quantity of crystalline insulin. The determinations are recorded in Table II, together with those of other investigators. Our results for cystine nitrogen are considerably higher than the values obtained by other workers recorded in Table II, but are in agreement with the more recent values reported by Jensen and Wintersteiner (19). In view of the fact that a variable quantity of cystine is precipitated with the basic amino acids (20), all the values reported in the present research have been calculated on a cystine-free basis.

An experiment has also been described in which crystalline insulin was slowly hydrolyzed with crystalline pepsin. At various stages before the complete destruction of the potency, samples were removed for biological assay and for determination of the nitrogen distribution. The results of these analyses have been presented in Table III. It will be noticed that in determining the nitrogen distribution in samples of the peptic digest, nitrogen could be detected in the distillate which was collected during the removal of the hydrogen chloride. This was not observed in the experiments with amorphous or crystalline insulin.

In the digestion experiment, a small sample of the insulin solution was removed before adding the pepsin. This sample was subject to the same conditions of heat and acidity as the larger quantity of insulin. The fact that there was no decrease in the potency of this control sample showed that the destruction of the physiological activity which was observed in the digest was due to peptic action and not to acid hydrolysis or prolonged heating. As is shown by Table IV, the destruction of the potency proceeded at a much greater rate than did the hydrolysis of the protein. This is in accord with the results of previous investigators. In our work, the protein fraction in each sample was precipitated in 3.3 per cent trichloroacetic acid. The nitrogen in the non-protein fractions was then determined by the micro-Kjeldahl method and the extent of hydrolysis calculated. Although this concentration of trichloroacetic acid is greater than some other workers have used, it was found to be both necessary and sufficient for the complete precipitation of the fraction responsible for the physiological activity. One cannot be assured that *only* physiologically active material was precipitated. The inclusion of an inactive fraction would, of course, result in a low value for the per cent hydrolysis reported in Table IV.

With respect to the determinations reported in Table III, some observations can be made. The values determined for the humin nitrogen are very low. This is probably due to the fact that insulin contains no tryptophane. No relation was found to exist between the physiological activity and the cystine content as determined by the method of Folin and Marenzi or of Sullivan. However, in view of the fact that these methods as applied in the present research will not differentiate between cystine and cysteine, it cannot be said with finality that sulfur-containing amino acids are not concerned in the inactivation of insulin by pepsin. A higher cystine content was indicated by the Sullivan method than by that of Folin and Marenzi. We can offer no explanation for this discrepancy. The composition of the standard cystine solution used for Folin's method and that used for Sullivan's method was checked by the use of a common procedure and found to be satisfactory. Microchemical analyses showed that there was no significant change in the sulfur content of the material during hydrolysis.

The arginine values are of interest in view of the report that guanidine derivatives possess hypoglycemic properties (21). Our work, however, showed no relation between the arginine content and the potency of the various samples. From Table III it is apparent that arginine has not been removed from the protein fraction during the early stages of peptic hydrolysis. Similar results have been obtained by Torbet and Bradley, using casein (22).

There has been a steady increase in the lysine content of the protein fraction. Reference to Table IV shows that this increase was greater than can be calculated from the extent of hydrolysis. The only explanation which can be offered is that the increased hydrolysis resulted in some structural change which permitted the determination of this increased quantity of lysine.

In the present research we have found that tyrosine is liberated in the early stages of peptic hydrolysis of insulin. Torbet and Bradley have reported that such is also the case in the peptic hydrolysis of casein (22). By absorption measurements, Kuhn, Eyer, and Freudenberg (7) have shown that tyrosine is of importance in a consideration of the structural formula of insulin. Our work has shown that the tyrosine content of crystalline insulin decreases as the activity of the hormone is destroyed by pepsin. To be sure, this decrease is not proportional to the reduction in physiological activity, but it is not to be expected that all the tyrosine in insulin is concerned with the hypoglycemic properties of the substance.

From a consideration of proposed formula weights for insulin, Freudenberg has drawn attention to the fact that the formula for insulin probably contains 14 molecules of tyrosine, only a few of which may be physiologically important. Such a view would be in accordance with the tyrosine determinations in the present paper. On the other hand, there is the work of Jensen and Evans (23) in which it was found that, in the case of the destruction of insulin with alkali, the tyrosine content is not affected. This statement, however, does not necessarily conflict with the present findings since two widely different processes of inactivation are concerned. Moreover, in the case of the experiments with alkali the tyrosine estimations were only made with difficulty.

The crystallization experiments described in this paper are of interest. Although these experiments were conducted by the best methods which have been developed for the crystallization of insulin, no rhombohedral crystals characteristic of insulin could be obtained from Samples B, C, or D. This was surprising, since amorphous insulin of the same unitage as Sample B (19.8 units per mg.) would yield large quantities of these crystals. Apparently this comparatively slight destruction of the physiological activity by peptic hydrolysis resulted in a change in the constitution of insulin of such a nature that its ordinary properties of crystallization were destroyed. However, in attempts to crystallize these products, needle-like crystals were obtained.

SUMMARY

A method has been described for the determination of the nitrogen distribution in small quantities of insulin. This method has been applied to the products of peptic digestion of crystalline insulin. The physiological activity of samples of the digest has been correlated with the nitrogen distribution. It was found that a loss in physiological activity was accompanied by a decrease in the tyrosine content. No significant variation in the quantity of cystine could be detected. An increase of lysine was observed. A comparatively slight destruction of the physiological activity of crystalline insulin by peptic hydrolysis yielded a product from which the rhombohedral crystals characteristic of insulin could not be obtained.

BIBLIOGRAPHY

1. du Vigneaud, V., Jensen, H., and Wintersteiner, O., *J. Pharmacol. and Exp. Therap.*, **32**, 367 (1927–28).
2. Abel, J. J., Geiling, E. M. K., Rouiller, C. A., Bell, F. K., and Wintersteiner, O., *J. Pharmacol. and Exp. Therap.*, **31**, 65 (1927).
3. Freudenberg, K., and Dirscherl, W., *Z. physiol. Chem.*, **202**, 192 (1931).
4. Dirscherl, W., *Z. physiol. Chem.*, **180**, 217 (1929).
5. Freudenberg, K., Dirscherl, W., and Eyer, H., *Z. physiol. Chem.*, **202**, 128 (1931).

6. Freudenberg, K., and Eyer, H., *Z. physiol. Chem.*, **213**, 226 (1932).
7. Kuhn, W., Eyer, H., and Freudenberg, K., *Z. physiol. Chem.*, **202**, 97 (1931).
8. Charles, A. F., and Scott, D. A., *Tr. Roy. Soc. Canad.*, sect. 5, **24**, 95 (1930).
9. Freudenberg, K., Dirscherl, W., Eichel, H., and Weiss, E., *Z. physiol. Chem.*, **202**, 159 (1931).
10. Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, **43**, 527 (1930).
11. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
12. Sullivan, M. X., *Pub. Health Rep.*, *U. S. P. H. S.*, suppl. 78 (1929).
13. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15 (1911–12).
14. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 89 (1929).
15. Rosedale, J. L., and da Silva, G. A., *Biochem. J.*, **26**, 369 (1932).
16. Tompsett, S. L., *Biochem. J.*, **25**, 2014 (1931).
17. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 109 (1929).
18. Winterstein, E., *Z. physiol. Chem.*, **34**, 153 (1901–02).
19. Jensen, H., and Wintersteiner, O., *J. Biol. Chem.*, **98**, 281 (1932).
20. Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **21**, 247 (1927).
21. Frank, E., Nothmann, M., and Wagner, A., *Klin. Woch.*, **5**, 2100 (1926).
22. Torbet, V., and Bradley, H. C., *J. Biol. Chem.*, **92**, lxxvii (1931).
23. Jensen, H., and Evans, E. A., *Z. physiol. Chem.*, **209**, 134 (1932).

THE INSULIN CONTENT OF THE PANCREAS IN CATTLE OF VARIOUS AGES

By A. M. FISHER and D. A. SCOTT

(*From the Connaught Laboratories, University of Toronto, Toronto, Canada*)

(Received for publication, June 1, 1934)

In attempting to estimate the insulin content of pancreas, there are many factors to be considered. In the first place, the amount of insulin may vary with the species of animal from which the pancreas is obtained. For example, it is generally believed that, per gm. of tissue, fish pancreas is much richer in insulin than ox pancreas. Secondly, the insulin content of the pancreas may vary with the season of the year. It has been our experience, in using beef glands, that the best yields of insulin are obtained during the winter months. Thirdly, since insulin is readily destroyed by proteolytic enzymes, fresh pancreas should be a better source of insulin than material which has been kept for some time before extraction. However, one of the most important factors is the method of extraction and purification of the insulin. Jephcott (1) has shown that the concentration of alcohol, the quantity and kind of acid, and the temperature during extraction all have a bearing on the amount of insulin which may be obtained from ox pancreas. Another factor is the age of the animal from which the pancreas is obtained, and it is this problem that we have investigated in the present research. We have experimented with the pancreas of one species only, namely the ox. All the glands were collected at the same season of the year (January 23 to February 7). Further, the time elapsing between the killing of the animals and the extraction of the insulin was the same in all age groups. A standard method of extraction and purification of the insulin was used.

EXPERIMENTAL

In the present investigation an attempt was made to determine the insulin content of the pancreas of cattle of the following ages:

fetal calves under 5 months; fetal calves 5 to 7 months; calves 6 to 8 weeks (still being fed with milk); cattle approximately 2 years old; cows 9 years and older; pregnant cows 7 years and older. In all cases, with the exception of the pancreas of fetal calves, the same procedure was followed in obtaining the pancreas and in the purification of the insulin. The method for each age group was as follows: Within ¾ hour after the removal of the glands, or about 1¾ hours after the death of the cattle, the pancreases of twelve to sixteen animals of the same age group were collected, minced, and thoroughly mixed. Three 25 gm. samples from each age group were weighed. To each of these, 100 cc. of extraction liquid were added. This liquid consisted of 750 cc. of absolute ethyl alcohol, 250 cc. of distilled water, and 15.0 cc. of concentrated hydrochloric acid. The insulin in each sample was extracted by shaking the acid-alcohol and pancreas for 2 hours at 37°. The residues were then removed by filtration through a double layer of cheese-cloth and extracted again with 100 cc. of the acidic ethyl alcohol for 1 hour. After the filtration of the second extractions, the first and second filtrates of each sample were combined and concentrated ammonium hydroxide added to each until the reaction was distinctly alkaline to litmus. The precipitates were centrifuged off and the volumes of the supernatant liquids measured. 10.0 cc. aliquots of these solutions were pipetted into three 50 cc. centrifuge tubes and the insulin in each was precipitated by adding 15 cc. of absolute ethyl alcohol and 25 cc. of ether. It was found from control experiments that this procedure precipitated the insulin quantitatively. The mixtures were placed in the refrigerator for 12 hours. They were then centrifuged and the precipitates were dried *in vacuo*. Each weighed approximately 15 mg. In view of the fact that a large number of samples were prepared in a comparatively short time, it was necessary to store some of the dried precipitates *in vacuo* for 3 or 4 weeks until each sample could be assayed. Preliminary experiments showed that this caused no loss of potency.

When working with the pancreases of fetal animals, it was necessary to vary the procedure slightly owing to the small size of the glands and the limited number which could be obtained at one time. In view of this, the three samples from each of these age groups were not extracted on one day, as described above for the older animals. Thus, with the fetal pancreas, it was necessary to adopt the following method. If, in the course of an hour's killing, three or more fetal calves of approximately the same age were obtained, the pancreatic glands were removed. These were minced and processed as one lot. The minced glands were weighed and the acid-alcohol added, 4.0 cc. for each gm. of pancreas. The extraction and purification were then conducted in a manner similar to that described for pancreas from the older animals. As occasion afforded, two additional lots of fetal pancreases of the same age group were collected and processed by the same method. The insulin precipitates obtained in each of these experiments were assayed independently, as described below.

Method of Assay

The insulin content of each of the eighteen samples of dried powder prepared by the process already described was determined by the mouse method of assay (2) in the following manner. The powder from each sample was dissolved in 40.0 cc. of isotonic saline to which sufficient hydrochloric acid had been added to adjust the reaction of the insulin solution to pH 2.5. An approximate indication of the activity of this solution was obtained by injecting 0.25 cc. quantities of it into twenty-five mice, and the convulsion rate compared with that obtained when similar quantities of a standard insulin solution were injected into the same number of mice. From the result of this test, the solution of unknown activity was diluted so that in subsequent tests approximately the same number of convulsions occurred among the mice injected with the solution of unknown strength as among those injected with the standard solution. To determine the potency of any sample, the solution was assayed four times against a standard insulin. The total number of mice used in such a test was 200. The average value of the four assays is recorded in Table I, and is expressed as units of insulin per gm. of pancreas. The average value of three samples is taken as the insulin content of the pancreas in each age group. This value is also incorporated in Table I.

DISCUSSION

The values in Table I show the comparative amounts of insulin which can be extracted from the pancreas of cattle of various ages.

Since, in carrying out the experimental work, every effort was made to have only one variable, namely age, we feel that the results obtained give a fairly accurate indication of the relative insulin content of the pancreas of the various age groups. It is evident from Table I that fetal pancreas is very rich in insulin. This is particularly true for fetal calves under 5 months, those on which the growth of hair has not commenced. In this group it will be noticed that the yields of the three experiments vary considerably. This is not surprising when it is remembered that in the case of fetal pancreas the glands were collected on three different occasions. Thus an error of a few weeks in the estimation of the age of these calves would result in an inaccurate determination of the insulin content for the age group in question. If the average yield of insulin per gm. of pancreas in the various groups be plotted against age, it will be noticed that the yield of insulin decreases very rapidly with age, until about 7 months after birth. After this time, increasing age causes a much less marked decrease in the yield of insulin. It is interesting to recall that fetal pancreases were used as the source of insulin in the early work on the preparation of insulin by Banting and Best (3).

TABLE I

Insulin Content of Pancreases of Cattle of Various Ages

Description of cattle	Results of biological assays (international units per gm. pancreas)			
	Sample 1	Sample 2	Sample 3	Average
Fetal calves, under 5 mos.	29.2	38.8	31.7	33.2
" " 5–7 mos.	23.2	24.9	21.1	23.1
Calves, 6–8 wks. (milk diet)	12.8	10.4	10.9	11.4
Cattle, 2 yrs.	3.9	6.1	4.5	4.8
Cows, 9 yrs. and older	1.7	2.0	1.8	1.8
" 7 " " " (pregnant)	2.0	2.2	2.3	2.2

The large yields of insulin obtained from fetal pancreas are of interest when considered in conjunction with the enzyme content of fetal animals. Ibrahim (4) found, in the case of human embryos, that pepsin and trypsin could be demonstrated in the digestive juices at about the 4th month, and that erepsin was present at the 5th month. Sampson (5) showed that there was no evidence of proteolytic activity in fetal pig pancreas until about half the gestation period had elapsed. Since insulin is destroyed by proteolytic enzymes, the low concentration of these enzymes in fetal animals may partially explain the large yields of insulin obtained from this pancreas. There are other explanations which might be advanced. The evidence of the transfer of an insulin-like material from fetal animals to the depancreatized mother is still controversial. Carlson and Drennan (6) found that if a bitch was depancreatized in the later stages of pregnancy, it showed no signs of diabetes. Removal of the pups by cesarean section, however, was followed by symptoms of diabetes. Similar findings were also reported by Aron, Stulz, and Simon (7). On the other hand, pregnancy did not decrease the insulin requirement of the depancreatized dogs observed in Macleod's laboratory (8), and most of the clinical evidence is in line with this finding. However, since there is a possibility of a mechanism providing for the transfer of an insulin-like substance between mother and fetus, we decided to determine whether or not the high insulin content of fetal pancreas was accompanied by a change in the amount of insulin contained in the pancreas of the mother. Our results with the pancreatic glands of pregnant cows over 7 years of age indicate that the insulin content of the pancreas of the mother is not affected by pregnancy. In this group the yields of insulin are slightly greater than for 10 year-old cows. This difference, we believe, is due to the difference in the age groups rather than to pregnancy.

SUMMARY

1. It has been shown that the amount of insulin which can be extracted per gm. of beef pancreas varies greatly with the age of the animal. Fetal beef pancreas is relatively rich in insulin.

2. In cows over 7 years of age, pregnancy would not appear to affect the amount of insulin in the pancreas.

3. An improved method for the preparation of experimental quantities of insulin from bovine pancreas has been described.

We wish to record our thanks to Canada Packers Limited for their cooperation in this investigation, and to the Federal Inspectors for making a careful estimation of the age of the animals.

BIBLIOGRAPHY

1. Jephcott, C. M., *Tr. Roy. Soc. Canad.*, sect. 5, **25**, 183 (1931).
2. Culhane, K., Marks, H. P., Scott, D. A., and Trevan, J. W., *Biochem. J.*, **23**, 397 (1929).
3. Banting, F. G., and Best, C. H., *J. Lab. and Clin. Med.*, **7**, 464 (1921–22).
4. Ibrahim, J., *Biochem. Z.*, **22**, 24 (1909).
5. Sampson, J. J., *J. Biol. Chem.*, **38**, 345 (1919).
6. Carlson, A. J., and Drennan, F. M., *Am. J. Physiol.*, **28**, 391 (1911).
7. Aron, M., Stulz, E., and Simon, R., *Compt. rend. Soc. biol.*, **89**, 571 (1923).
8. Macleod, J. J. R., Carbohydrate metabolism and insulin, London and New York (1926).

THE PREPARATION OF SODIUM TUNGSTATE FREE FROM MOLYBDATE, TOGETHER WITH A SIMPLIFIED PROCESS FOR THE PREPARATION OF A CORRECT URIC ACID REAGENT (AND SOME COMMENTS)

By OTTO FOLIN

(*From the Biochemical Laboratory of the Harvard Medical School, Boston*)

(Received for publication, June 7, 1934)

This contribution is essentially only a supplement to my 1933 (1) paper on the determination of uric acid, yet it would be misleading to say so in the title. For many years I have labored, off and on, with the problem of preparing a uric acid reagent "completely free from phenol reagent," and each time I succeeded and concluded the problem was solved, only to find at a later date, with different chemicals, that the problem had not been solved. Each time I assumed, probably correctly enough, that I had not, after all, succeeded in removing all the molybdate present in the tungstate, but when this happened again a few months after the publication of my 1933 paper, it seemed necessary definitely to settle the problem by working only with sodium tungstate known to be completely free from molybdate. I therefore set about once more to try to prepare such a tungstate. One theoretically important reason for trying to prepare such a tungstate was the finding reported by Wu (2) that the uric acid reagent of Folin and Denis contains two different chromophorically active phosphotungstic acids having the same composition, except for their different water content, yet differing markedly in their chromophoric values. If Wu's observations are correct, the old uric acid reagents should in fact contain no less than three different chromophorically active ingredients, namely the A and B compounds described by Wu, and in addition some phenol reagent due to the presence of molybdate. And it has now become clear that such is indeed the case.

Preparation of Sodium Tungstate Free from Molybdate—The natural but unfortunate mistake heretofore made in trying to remove the last traces of molybdate from sodium tungstate has been the endeavor to convert the molybdate into *insoluble* sulfides. In the process described below the molybdates are converted into highly colored sulfomolybdates which are very soluble in alcohol as well as in water, and which therefore can be separated very readily from the tungstate, which is insoluble in alcohol. The process is as follows:

Dissolve 1 kilo of sodium tungstate in 2 liters of water and add hydrochloric acid (dilution 1:1), *slowly* and with stirring, until the solution is neutral to litmus paper. An acid reaction is undesirable.

Transfer the solution to a large flask or bottle. Pass H_2S into the solution in a moderately rapid stream for 15 to 20 minutes. Stopper the container and let stand overnight.

Transfer to large beakers and add, *very slowly* at first and with constant stirring, about $\frac{2}{3}$ volume of alcohol. The paratungstate is precipitated and the colored sulfomolybdates remain in solution. Let stand till the next day; decant and filter, with suction, on a large Buchner funnel; wash with 50 per cent alcohol until the filtrate is colorless.

Transfer the precipitate to a 4 liter beaker, add 1.5 liters of water and about 2 cc. of bromine, and stir for a few minutes. Then heat over a burner and continue the stirring until all the surplus bromine has been removed. (The bromine treatment is for the purpose of oxidizing such traces of sulfotungstates as are likely to be present.) Continue the heating and add clear saturated sodium hydroxide solution until the solution gives a *permanent* and fairly strong reaction with phenolphthalein paper. Cool. If the solution is turbid, filter. Then precipitate as before, with alcohol, and dry.

The sodium tungstate thus obtained gives a moderate, permanently alkaline, reaction with phenolphthalein; its solutions are clear, and it does not give the slightest trace of pink color in the potassium xanthate test for molybdate described by Folin and Trimble (3). Photographed spectrum analyses, kindly made for me by Professor F. A. Saunders of the Department of Physics, Harvard University, have shown that the product is completely free from molybdenum.

This pure sodium tungstate is now made on a large scale by the

Mallinckrodt Chemical Company under the supervision of Dr. A. D. Alt, and samples of their product have been found completely free from molybdate. But the application of the potassium xanthate test to every new batch of bought sodium tungstate should of course not be omitted.

Simplified Method for Preparation of Perfect Uric Acid Reagent—Transfer 100 gm. of sodium tungstate (free from molybdate) to a 500 cc. Florence flask. Mix 32 to 33 cc. of 85 per cent phosphoric acid with 150 cc. of water. Pour the resulting solution on to the tungstate and mix. Add a few pebbles and boil *very gently* over a microburner for 1 hour. Loss of liquid during the boiling is prevented by using, as a condenser, a funnel holding a 200 cc. flask filled with cold water. At the end of the boiling period decolorize with a little bromine water, boil off the excess bromine, cool, and dilute to 500 cc.

If the reagent so obtained is not perfect (in other words if it gives a blank with Merck's urea-cyanide or with urea-cyanide plus tyrosine), add 3 to 5 gm. of sodium tungstate (but no more) and boil for another 10 to 15 minutes, then cool, and decolorize as before. The addition of a little extra tungstate and the short second boiling can also be made without first testing the reagent for a blank.

This last supplementary procedure as well as the direction to use only 32 to 33 cc. of phosphoric acid for 100 gm. of tungstate is based on observations which confirm Wu's findings of the two different chromophorically active phosphotungstic acids, already referred to. Wu's hypersensitive A reagent behaves more or less like a phenol reagent and it is largely because of the sporadic formation of this compound that the preparation of a correct reagent, containing only the phosphotungstic acid B, has proved so elusive. The range of acidity below which little or no active reagent is formed, and the acidity yielding the A compound as well as the desired B compound is very narrow. Attempts to standardize the correct range of acidity by titration or by colorimetric pH determinations have not proved practically useful.

Concluding Comments

This is presumably my last paper on the preparation of the uric acid reagent and I hope that the method for the determination of uric acid described last year is also final.

With regard to determination by the "indirect" or silver precipitation method described in that same paper, a few additional remarks are perhaps necessary. Those who wish to use that method should first apply it to 5 cc. of the standard uric acid solution so as to make sure that they can secure a 100 per cent recovery with as little as 0.02 mg. of uric acid. In the course of such preliminary check work it will be found that losses of uric acid are avoided only by working, with what will seem to some, great speed. Slow work or any undue delay will result in some destruction of uric acid, independently of exposure to light, and a very little destruction will appear large when expressed as per cent. It is particularly important that the silver precipitate be dissolved quickly and completely in the urea-cyanide solution. By the help of a suitable (not too fine) stirring rod the solution of the silver precipitate can be attained in a few seconds. It should be remembered that any losses found are almost certainly due to destruction of uric acid and not to incomplete precipitation.

While the uric acid determination now appears to be practically reliable and satisfactory, there remain many theoretically interesting problems requiring further investigation. One of these is the fact that probably less than 10 per cent of the color obtained in the modern forms of the method represents the direct reduction of the reagent by the urate. The other 90 per cent (more or less) represents indirect or induced reduction due to the presence of the cyanide. The latter reduction is rather more specific for uric acid than the directly produced smaller fraction. The indirect reduction is subject to great variations in magnitude according to the quality of the uric acid reagent, the quality of the cyanide, and other modifying factors, such as the degree of alkalinity, the presence of carbonate, of amino compounds, of some phenol products, etc.

Because of these obscure factors, the colorimetric method for the determination of uric acid represents probably the most complex reaction that we have in the whole field of practical colorimetry.

BIBLIOGRAPHY

1. Folin, O., *J. Biol. Chem.*, **101**, 111 (1933).
2. Wu, H., *J. Biol. Chem.*, **43**, 198 (1920).
3. Folin, O., and Trimble, H., *J. Biol. Chem.*, **60**, 474 (1924).

COMPARATIVE STUDIES OF THE METABOLISM OF AMINO ACIDS

VI. THE RATE OF ABSORPTION OF LEUCINE, VALINE, AND THEIR ISOMERS FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT*

By BARBARA W. CHASE AND HOWARD B. LEWIS

(*From the Department of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor*)

(Received for publication, May 31, 1934)

In previous studies from this laboratory, the method of Cori has been used in the determination of the rate of absorption from the gastrointestinal canal of the white rat of various amino acids, glycine, alanine, leucine, and glutamic acid (1), and of methionine (2). Wilson (3) and Sullivan and Hess (4) have applied the same technique to the study of the absorption of cystine and Berg and Bauguess (5) have investigated the absorption of tryptophane. The work here reported is concerned with the absorption of the three structurally isomeric leucines and two valines. A comparison of the rate of absorption of the racemic (*dl*-) and the naturally occurring optically active forms has also been made. The glycogen content of the liver after the administration of these amino acids was also determined.

EXPERIMENTAL

Male rats subjected to preliminary fasting periods of 24 hours were selected so that the weights after fasting were approximately the same, 120 to 135 gm. The methods of preliminary treatment of the animals, administration of the amino acids, and of analysis of the gastrointestinal contents for amino acid nitrogen and of the liver for glycogen were similar to those previously reported (1).

---

* A preliminary report of part of this work was presented before the American Society of Biological Chemists at Cincinnati (Chase, B. W., *J. Biol. Chem.*, 100, xxvii (1933)).

Unless otherwise noted, the absorption of the amino acid was allowed to proceed over a period of 3 hours.

All the amino acids administered were analyzed both for total

TABLE I

Rate of Absorption of Sodium Salts of Isomeric Leucines during Periods of 3 Hours

Substance fed	Rat No.	Weight after fasting	Amount fed	Rate of absorption per hr. calculated in terms of		
				Nitrogen	Amino acid	
		gm.	*mg. per 100 gm. rat*	*mg. per 100 gm.*	*mg. per 100 gm.*	*m.-eq. per 100 gm.*
l-Leucine	53	121	185	4.5	42	
	54	116	194	5.2	49	
	55	148	152	4.6	43	
	58	127	189	5.1	47	
	59	108	214	5.1	47	
	61	135	167	4.3	40	
Average				4.8 (4.6)*	45 (43)*	0.34 (0.33)*
dl-Leucine	91	120	145	4.3	40	
	122	120	191	4.6	43	
	123	116	197	5.9	55	
	124	117	195	4.7	44	
	125	115	180	4.5	42	
	127	132	178	5.1	47	
	128	122	193	5.0	47	
	129	130	181	5.1	47	
	130	125	188	4.9	45	
Average				4.9	45	0.34
d-Isoleucine	62	114	201	3.7	34	
	63	121	188	2.9	27	
	64	120	190	3.0	28	
	65	120	191	3.8	36	
	103	130	187	4.2	39	
	107	127	185	4.1	38	
	108	120	196	4.2	39	
	109	127	185	3.7	34	
Average				3.7	34	0.26

* Values obtained by Wilson and Lewis (1).

TABLE I—*Concluded*

Substance fed	Rat No.	Weight after fasting	Amount fed	Rate of absorption per hr. calculated in terms of		
				Nitrogen	Amino acid	
		gm.	*mg. per 100 gm. rat*	*mg. per 100 gm.*	*mg. per 100 gm.*	*m.-eq. per 100 gm.*
dl-Isoleucine	116	129	203	3.9	36	
	117	129	203	3.1	28	
	118	126	181	3.6	33	
	119	120	190	3.7	34	
	120	120	190	4.0	37	
	121	117	195	3.6	33	
	131	147	180	3.6	33	
	132	150	181	4.6	43	
	133	145	182	4.0	37	
Average...........................				3.8	35	0.27
dl-Norleucine	66	120	214	3.5	32	
	68	133	193	3.6	33	
	69	126	203	3.8	36	
	111	118	204	4.4	41	
	112	138	175	3.7	34	
	113	115	210	3.8	36	
	134	129	221	4.2	39	
	135	127	229	3.3	30	
	137	130	219	3.7	34	
Average...........................				3.8	35	0.27

nitrogen (Kjeldahl-Gunning) and for amino nitrogen (Van Slyke gasometric method). It is of interest to note that for the complete reaction of the α-amino group of isovaline with nitrous acid in the Van Slyke gasometric method, 15 minutes were necessary rather than the usual 3 to 5 minute period for the reaction of α-amino groups. Because of the insolubility of valine and the leucines, it was necessary to administer the sodium salts in order to obtain a sufficiently high concentration of the amino acid to permit the study of absorption. Isovaline (α-amino-α-methylbutyric acid) was sufficiently soluble to make possible administration of the amino acid itself, but in order to make the results of the entire series comparable, the sodium salt of this acid was fed also.

The normal control values for the amino nitrogen content of the gastrointestinal canal of the young rat after short fasts as determined in a series of twenty-one animals averaged 7.9 mg. with individual determinations ranging from 5.0 to 11.3 mg. as previously reported (2). This average control value has been used as a correction for all the experimental values reported in Tables I and II.

The results obtained with the three structurally isomeric leucines are presented in Table I. No significant difference between the rates of absorption of the naturally occurring amino acid and the *dl* form was observed with either leucine or isoleucine. It is of interest to note that the results with *l*-leucine in the present series agree well with those obtained previously in this laboratory (1). The rates of absorption of isoleucine and norleucine were, however, significantly less than that of leucine. Although the difference was not marked, we believe that it is beyond the range of the experimental error of the method. In only two animals (Rats 111 and 132) were values found higher than the minimal values obtained with either *l*- or *dl*-leucine. The rates of absorption of isoleucine and norleucine were essentially identical.

The rates of absorption of both *d*- and *dl*-valine were essentially the same (Table II) and were greater than that of leucine. Isovaline (α-amino-α-methylbutyric acid) when administered as the sodium salt was very slowly absorbed, the absorption coefficient calculated in milli-equivalents being 0.14 as compared with 0.40 and 0.37 for *dl*- and *d*-valine[1] respectively. The absorption in 3 hours was so slight as to make the absolute values subject to considerable error since the amount absorbed was in many cases less than the blank determination of the amino acid nitrogen content of the tract of the normal fasting animal for which correction was made. The absorption coefficient of the sodium salt of isovaline was so low that another salt (potassium) was studied; the results obtained, however, did not differ from those discussed. In sharp contrast to its salts, the free acid, isovaline, was absorbed readily, the values obtained being greater than any of those in the present series. This is difficult to interpret. Similar but less

---

[1] A series of four animals fed with *dl*-valine over absorption periods of 2 hours each gave absorption coefficients of 5.4, 45, and 0.38 calculated as mg. of nitrogen, mg. of amino acid, and milli-equivalent respectively.

TABLE II

Rate of Absorption of Sodium Salts of Isomeric Valines during 3 Hour Periods

Substance fed	Rat No.	Weight after fasting	Amount fed	Rate of absorption per hr. calculated in terms of		
				Nitrogen	Amino acid	
		gm.	*mg. per 100 gm. rat*	*mg. per 100 gm.*	*mg. per 100 gm.*	*m.-eq. per 100 gm.*
d-Valine	32	114	248	6.1	51	
	33	120	237	6.6	55	
	34	105	271	5.5	46	
	35	102	272	6.1	50	
	72	113	235	6.0	50	
	74	149	180	5.0	41	
	80	125	226	4.5	38	
Average				5.7	47	0.40
dl-Valine	9	134	173	5.1	42	
	11	124	148	5.6	46	
	15	95	261	5.4	45	
	16	128	202	4.8	40	
	25	110	236	5.4	45	
	27	107	244	4.8	40	
	28	131	215	5.5	46	
	29	109	259	5.8	48	
	30	135	208	5.8	48	
Average				5.3	44	0.37
dl-Isovaline	41	139	145	2.9	24	
	42	172	116	2.2	18	
	43	126	153	1.2	10	
	44	125	155	2.7	23	
	51*	119	171	1.2	10	
	52*	117	174	1.8	15	
Average				2.0	16	0.14
dl-Isovaline†	45	130	161	5.0	42	
	46	123	170	6.3	53	
	75	115	222	6.3	53	
	76	125	205	7.2	59	
	77	112	228	7.4	62	
Average				6.4	54	0.46

* The potassium salt was fed.

† The free amino acid was fed.

striking differences between the absorption of alanine and its sodium salt were obtained previously (1), while in the case of glycine, absorption was increased by the presence of sodium in the molecule (1).

The results reported here, which show in the case of leucine, isoleucine, and valine, no difference in the rate of absorption between the naturally occurring optical isomer and the racemic form, are not in accord with the findings of Berg and Bauguess (5) for *l*- and *dl*-tryptophane. These investigators reported absorption coefficients of 0.284 and 0.328 milli-equivalents for the *l* and *dl* forms respectively. In our opinion, these variations are so slight as to be practically within the limits of the experimental error of the method. It may also be noted that *d*-, *l*-, and *dl*-lactic acid were absorbed at the same rate (6), a finding in harmony with the older observations of Dakin (7) in a study on the isolated loops of the intestine.

In summary, the results of the work in this laboratory and elsewhere indicate that in periods of 3 hours the absorption coefficients of the sodium salts of the amino acids calculated as milli-equivalents per 100 gm. per hour are as follows: glycine, 0.84; alanine, 0.54; cystine, 0.44 (calculated as cysteine); glutamic acid, 0.41; valine, 0.40; methionine, 0.36; leucine, 0.34; isoleucine and norleucine, 0.27; and isovaline, 0.14. No convincing proof of the influence of the stereochemistry of the amino acid on its absorption coefficient has as yet been obtained.

Determinations of the glycogen content of the liver failed to afford any evidence of glycogen formation from any of the amino acids of the present series[2] under the experimental conditions used. Such results were not unexpected in view of the fact that leucine, isoleucine, and valine are not considered to be gluconeogenic. Norleucine, however, has been shown to give rise to glucose in the phlorhizinized dog. No information as to the gluconeogenic properties of isovaline is to be found in the literature so far as is known to us.

---

[2] No glycogen determinations were made after the feeding of *d*-isoleucine.

BIBLIOGRAPHY

1. Wilson, R. H., and Lewis, H. B., *J. Biol. Chem.*, **84**, 511 (1929).
2. Chase, B. W., and Lewis, H. B., *J. Biol. Chem.*, **101**, 735 (1933).
3. Wilson, R. H., *J. Biol. Chem.*, **87**, 175 (1930).
4. Sullivan, M. X., and Hess, W. C., *Pub. Health Rep., U. S. P. H. S.*, suppl. 89 (1931).
5. Berg, C. P., and Bauguess, L. C., *J. Biol. Chem.*, **98**, 171 (1932).
6. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **81**, 389 (1929).
7. Dakin, H. D., *J. Biol. Chem.*, **4**, 437 (1908).

CHANGES OF TOTAL LIPID AND IODINE NUMBER OF BLOOD FAT IN ALIMENTARY LIPEMIA

By WILLIAM R. WILSON and JAMES P. HANNER

(*From the Department of Pediatrics of Yale University School of Medicine and the Pediatric Service of the New Haven Hospital, New Haven*)

(Received for publication, May 1, 1934)

This study was undertaken to determine some of the qualitative changes in the blood fat during alimentary lipemia. An increase in the blood lipids as a result of ingestion of fat has been repeatedly demonstrated (Bloor (1), Hiller, Linder, Lundsgaard, and Van Slyke (2), Hejda (3), McClure and Huntsinger (4), Man and Gildea (5)). These workers are all essentially in agreement that the increase involves principally the fatty acid, with an inconstant small increase in the lipoid phosphorus and no demonstrable change in the cholesterol. The present experiments are designed to determine the character of the fatty acids which appear in plasma following ingestion of fats with widely different characteristics. For this purpose, cream, with an iodine number of 30 to 40 and cod liver oil, with an iodine number of about 155, were used.

EXPERIMENTAL

The fats were fed to convalescent patients aged 4 to 12 years who showed no demonstrable metabolic disturbance. The patient received supper at 5.30 p.m. and no breakfast. A control sample of blood was taken at 8.30 a.m., after which the cream or cod liver oil was given. In the experiments with cream, specimens were collected 2 and 4 hours after ingestion, after cod liver oil only one specimen was taken. On each occasion about 15 cc. of blood were withdrawn. The subjects took no food but were allowed water during the experiment. The specimens were allowed to clot, the clot broken with a stirring rod, and the serum pipetted off after centrifuging. All determinations were carried out on serum.

Methods

The sera were extracted with an alcohol-ether (alcohol 3 parts, ether 1 part) mixture, as described by Bloor. The serum (4 to 6 cc.) was pipetted slowly with continuous rotation into an Erlenmeyer flask containing about 60 cc. of the mixture of alcohol and ether. Several glass beads were added to prevent bumping and the extraction mixture was refluxed on the hot plate for an hour with an "underwriters" apparatus or a reflux condenser.[1] The extract was filtered through a fat-free filter paper into a 100 cc. volumetric flask, the protein precipitate was washed several times with small portions of the mixture of alcohol and ether, and the filtrate made up to volume. Aliquots were taken for the different determinations.

Lipoid phosphorus was determined by the method of Benedict and Theis (7) on the evaporated mixture of alcohol and ether digested with concentrated H_2SO_4 and superoxol, as suggested by Baumann (8). Cholesterol was determined by the colorimetric method of Sackett (9), the determination being based upon the green color formed by cholesterol in reaction with acetic anhydride and concentrated H_2SO_4. The fatty acid determination was carried out by the method of Stoddard and Drury as modified by Man and Gildea (6). The total lipid was determined by a gravimetric method developed in this laboratory. A description of the procedure follows.

Measure into a 125 cc. Erlenmeyer flask an aliquot of alcohol-ether extract equivalent to approximately 1 cc. of serum. Add 0.08 cc. (4 drops) of 0.1 per cent alcoholic solution of hydroquinone to prevent oxidation of unsaturated fats.[2] Evaporate the extract just to dryness, being sure to remove all the alcohol. Avoid overheating. The fat apparently is not altered by a temperature of 110° for a short time (at least 5 minutes). Suspend the residue in 2 to 5 cc. of distilled water. Add 1 drop of concentrated HCl (sp. gr. 1.19) to insure acidity and absence of soluble soaps. Extract with about 10 cc. of petroleum ether (b. p. 30–60°) by rotating on the hot plate until about 5 cc. of the solvent remain. After the layers have separated, transfer the supernatant fluid by small rubber bulb pipette to a tared weighing bottle. (Bottles which hold 15 to 20 cc. and weigh less than 20 gm. were used.) Repeat the extraction several times until the weighing bottle is almost full. Add 0.08 cc. of hydroquinone solution to the contents of the weighing flask. Evaporate the petroleum ether solution to dryness on a hot plate, avoiding explosive boiling. Allow the weighing flask to stand 2 hours in the balance room to come to constant weight. Weigh the flask and subtract the tare to obtain the number of mg.

---

[1] Refluxing for an hour is a modification suggested by Man and Gildea (6).

[2] This amount of hydroquinone will protect at least 12 mg. of linoleic acid and eliminates the necessity of carrying out the drying of the extract in an atmosphere of CO_2 or N_2. Olcott, in working with keratinase and precursors of vitamin B, found hydroquinone a very powerful antioxidant and suggested its use in this procedure, as it has no unsaturated bonds and does not interfere with the iodine reaction. (Olcott, H. S., personal communication.)

TABLE I

Test of Method with Theoretical Solutions

Experiment No.	Material used	Theoretical content	Weight obtained	Iodine No.	
				Theoretical	Obtained
		mg.	*mg.*		
1	Cod liver oil	28.7	28.3 28.6	158	156
2		14.35	14.2 14.0	158	154
3	Linseed oil	26.49	26.4 26.3	165	166
4		13.24	13.1 12.9	165	163
5		15.49	15.6 15.6	165	163.5
6	Linoleic acid-palmitic acid-cholesterol	14.96	14.9 14.7	103.6	102.9

In Experiments 1, 2, 3, and 4, the alcohol-ether extract was dried under CO_2. The petroleum ether extract was dried with hydroquinone for protection against oxidation. In Experiments 5 and 6, hydroquinone was used as the only protection against oxidation.

Four sets of duplicates in these experiments were weighed on both the Pregl and chainomatic balance, and all flasks agreed within 0.1 mg. on the two balances. The Pregl balance was used in the experiments reported, but the ordinary chainomatic laboratory balance is apparently adequate.

of fat in the amount of serum used. After being weighed, the fat is dissolved in 3 cc. of chloroform and transferred with three washings to a glass-stoppered 125 cc. Erlenmeyer flask and the iodine number determined by the method described by Yasuda (10).

TABLE II

Effect upon Blood Lipids of Feeding Fats of Widely Different Iodine Number

Case	Age	Material ingested	Fat	Time after feeding	Cholesterol	Lipoid P	Total lipid	Increase of total lipid	Total fatty acid	Increase of fatty acid	Iodine No.	
											Total lipid	Increment
	yrs.		*gm. per kg.*	*hrs.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*m.-eq.*	*m.-eq.*		
M. D.	13	Cream	4.0	0	288	8.3	800		14.3		125	
				2	300	9.0	816	16	15.1	0.8	121	
				4	320	11.7	925	125	17.4	3.1	110	46
R. S.	9	"	4.7	0	188	7.6	740		12.9		71	
				2	188	7.7	835	95	16.35	3.45	69	50
				4	188	7.7	1063	323	23.2	10.3	62	39
T. M.	8	"	4.1	0	150	5.4	333		7.85		99	
				2	150	5.4	450	117	11.6	3.75	83	54
				4	150	5.4	503	170	12.6	4.75	82	60
S. S.	6	"	5.6	0	188	6.6	506		9.8		87	
				2	188	6.8	688	182	15.9	6.1	78	55
				4	188	7.8	775	269	19.0	9.2	74	50
L. E.	6	Cod liver oil	2.8	0	283	11.0	683		10.34		94.5	
				2	271	11.0	735	52	12.04	1.7	97.4	135
A. R.	4	" " "	3.5	0			590		10.27		92.6	
				4			625	35	11.03	0.76	97.8	185
J. W.	6	" " "	5.0	0	200	8.8	570		9.3		89.1	
				4	200	8.8	720	150	14.3	5.0	95.8	118
D. P.	6	" " "	5.0	0	160	6.8	480		9.0		107.2	
				3	160	8.0	740	260	16.47	7.47	113.4	123

Determinations were carried out on known solutions of cod liver oil and linseed oil to test the efficacy of the hydroquinone in preventing oxidation of the fats when the solvents were dried. The results are shown in Table I. Earlier attempts to prevent oxidation with 0.02 mg. of hydroquinone were not successful. Hydroquinone is not carried over from the water suspension in the petroleum ether. The 0.08 mg. added to the petroleum ether extract is subtracted from the final weight. Aqueous solutions of glucose, sodium chloride, and hydrochloric acid were also extracted with petroleum ether and the residue weighed. In each instance none of these substances was carried over by the solvent. Bloor has used petroleum ether in the reextraction in his oxidative method and found it a less active but more selective solvent than the other lipid solvents. The evidence available indicates that the material reextracted by the petroleum ether represents completely and exclusively the lipid content of the blood serum.

Results

The results of the determinations carried out on blood from fasting and postabsorptive subjects are shown in Table II. The increases in the various constituents are similar to those found in other reports. The fasting values for total lipid and other constituents determined were as follows:

	Total lipid	Fatty acids	Lipoid P	Cholesterol	Iodine No. of total lipid
	mg. per 100 cc.	*m.-eq.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
No. of determinations	8	8	7	7	8
Average	588	10.47	7.8	208	95.7
Minimum	333	7.85	5.4	150	71
Maximum	800	14.3	11.0	288	125

Following the ingestion of cream there was an average increase in the total lipid of 222 mg. per 100 cc. and an average increase in the fatty acids of 6.8 milli-equivalents per liter. The changes in lipoid phosphorus and cholesterol were smaller and less regular, indicating that the increase affected principally, if not entirely, the neutral fat. The iodine number in each case decreased. The increases in blood lipid following ingestion of cod liver oil were smaller, probably because smaller amounts of the fat were administered, but were also principally, if not entirely, in the neutral fat. The total lipid underwent an average increase of 126 mg. per 100 cc. and the fatty acid increase averaged 3.7 milli-equivalents per liter. The iodine number in each case increased.

An attempt to calculate the molecular weight of the increase in fatty acid by dividing the mg. per 100 cc. increase of total lipid by the milli-equivalent increase of fatty acids gave irregular values. The errors inherent in the methods are such that large errors appear in indirect calculations of this type and no conclusions can be drawn.

The results of the determinations of the iodine number, however, are more definite. The iodine number of the increment was calculated by the formula, (increase in mg. of iodine absorbed per 100 cc. × 100) / (increase in mg. of fat per 100 cc.). The iodine number of the increment following the ingestion of cream (iodine number 30 to 40) ranged between 39 and 60, in each case representing a decrease in the iodine number of the blood fat. Following cod liver oil (iodine number 156 to 160), the iodine number of the increment was between 118 and 135, in each case representing an increase in the iodine number of the blood fat. The iodine number of the increment in these two groups is of the same order of magnitude as the ingested fat and suggests that the increment of fatty acid in the blood during alimentary lipemia is made up of the same fatty acids as the ingested fat. When lipoid phosphorus or cholesterol increases, allowance is made for this increase in the calculations.

DISCUSSION

The mechanism of fat absorption has recently been reviewed by Verzar (11). He reviews the process of digestion in the intestine, citing evidence that the neutral fat is hydrolyzed in the intestine and resynthesized in the intestinal mucosa. Sinclair (12) obtained evidence which he interpreted as indicating that the fatty acids of ingested fat are incorporated in the phospholipid of the intestinal mucosa and later released in the form of the neutral fat which makes up the bulk of the fat in the chyle in the postabsorptive state. His evidence that the phospholipid is an intermediary in the resynthesis is based upon an increase in the iodine number of the phospholipid fatty acid following ingestion of cod liver oil, and an indefinite decrease following ingestion of coconut oil (iodine number 10). A later paper (13) offers perhaps a better explanation for the changes in iodine number of the phospholipid in these experiments, as in the later experiments he demonstrates a marked tendency for the phospholipid of the body to take up unsaturated fatty acids, substituting them for saturated ones, and to retain them after unsaturated acids are removed from the diet. Verzar (11) does not attempt to explain the mechanism by which the fatty acids are absorbed and resynthesized. He quotes the studies of Munk on material obtained from a chylous fistula, showing that about 60 per cent of the fat absorbed is transmitted through the lacteals in the form of neutral fat.

Fatty acids are transported in the blood in three types of combinations—phospholipid, cholesterol ester, and neutral fat. The increase in phospholipid in alimentary lipemia is inconstant and, when it occurs, not great enough to account for any considerable proportion of the increase in fatty acid. Cholesterol transports fatty acids in the form of esters but the amount transported in this manner is also inadequate to account for a very large fraction of the increase in fatty acid in alimentary lipemia. Knudson (14) studied the cholesterol, both free and in ester form, in the blood during alimentary lipemia and found no change in the total cholesterol, but a slight increase in the proportion combined with fatty acids. This fraction is also inadequate to account for a substantial portion of the increase of fatty acid. These facts and the work of Munk indicate that neutral fat is the form in which most, if not all, of the absorbed fat is transported in the blood stream, either to the organs in which it is metabolized or to the depots of the body, where it is stored as neutral fat. The details of these processes are almost entirely unknown.

Anderson and Mendel (15) and others have demonstrated that the character of the food fat influences the type of fat stored in the fat depots of the body. The iodine number of the body fat of rats fed 60 per cent of their total caloric intake in the form of various fats approximated the iodine number of the food fat. This is further evidence that the increment of neutral fat in the blood stream following ingestion of fat is composed of the fatty acids contained in fat ingested.

SUMMARY

Alimentary lipemia was produced in four well children by administration of 40 per cent cream (iodine number 30 to 40) by mouth and in four children by administration of cod liver oil (iodine number 165). The calculated iodine number of the increment was between 39 and 60 in those fed cream and between 118 and 135 in those fed cod liver oil. These findings are interpreted as evidence that the neutral fat resynthesized by the intestinal mucosa and absorbed into the blood stream following ingestion of fat, is largely or entirely composed of the fatty acids contained in the fat ingested. A gravimetric method for determination of total lipid in the blood is also described.

BIBLIOGRAPHY

1. Bloor, W. R., *J. Biol. Chem.*, **24**, 447 (1916).
2. Hiller, A., Linder, G. C., Lundsgaard, C., and Van Slyke, D. D., *J. Exp. Med.*, **39**, 931 (1924).
3. Hejda, B., *Am. J. Med. Sc.*, **180**, 84 (1930).
4. McClure, C. W., and Huntsinger, M. E., *J. Biol. Chem.*, **76**, 1 (1928).
5. Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, **99**, 61 (1932–33).
6. Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, **99**, 43 (1932–33).
7. Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, **61**, 63 (1924).
8. Baumann, E. J., *J. Biol. Chem.*, **59**, 667 (1924).
9. Sackett, G. E., *J. Biol. Chem.*, **64**, 203 (1925).
10. Yasuda, M., *J. Biol. Chem.*, **94**, 401 (1931–32).
11. Verzar, F., *Nutrition Absts. and Rev.*, **2**, 441 (1933).
12. Sinclair, R. G., *J. Biol. Chem.*, **82**, 117 (1929).
13. Sinclair, R. G., *J. Biol. Chem.*, **96**, 103 (1932).
14. Knudson, A., *J. Biol. Chem.*, **32**, 337 (1917).
15. Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **76**, 729 (1928).

THE REACTION OF IODOACETIC ACID ON MERCAPTANS AND AMINES

By L. MICHAELIS and MAXWELL P. SCHUBERT

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 10, 1934)

Since Lundsgaard (1) discovered the effect of iodoacetic acid on the chemical processes attending muscle contraction, various other remarkable effects of this substance in biochemical processes have been encountered. The interpretation of this effect, especially after Dickens' publication on this matter, amounts to assuming a chemical interaction of iodoacetic acid and sulfhydryl groups according to the scheme

$$R\text{—}SH + ICH_2COOH = R\text{—}SCH_2COOH + HI$$

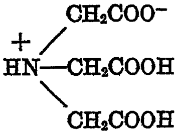
Dickens (2) was the first to describe the preparation of the S-carboxymethyl compounds of cysteine and glutathione, which we also had just prepared when Dickens' paper was published.

There can be no doubt that on the interaction of a sulfhydryl compound with iodoacetic acid this reaction occurs with great ease, and compounds of this kind will be described in the experimental part beside those prepared by Dickens.

It seems, however, not to be generally appreciated that iodoacetic acid also reacts quite easily with amino groups according to the equation

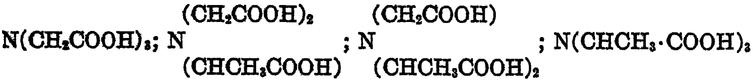
$$RNH_2 + 2ICH_2COONa + 2NaOH \rightarrow RN(CH_2COONa)_2 + 2NaI + 2H_2O$$

This reaction occurs with both aliphatic and aromatic amines and products of the action of iodoacetic acid on glycine, alanine, cystine, and *p*-phenylenediamine will be described. The product obtained from glycine and 2 moles of iodoacetic acid has also been obtained from ammonia and 3 moles of the halogen acid. This particular compound has already been prepared by Heintz (3). The remarkable feature of these reactions is the ease with which complete substitution of all the hydrogen of amino groups can be accomplished. The products are polycarboxylic acids as an example of which may be mentioned $N(CH_2COOH)_3$ obtained from either ammonia or glycine. Evidence is also presented that this can exist as a zwitter ion.



When an aqueous solution of this compound is potentiometrically titrated with standard alkali, two carboxyl groups are titrated in succession behaving as rather strong acids and a 3rd equivalent of NaOH is used up in the alkaline range to deionize the ammonia group. The third carboxyl group is ionized from the beginning and does not show up during titration with alkali. There is so far no evidence that quaternary ammonium salts are formed in any of these reactions.

The reaction described has been carried out with halogen acids other than iodoacetic acid and is probably general with halogenated fatty acids, though very likely the iodo compounds are more reactive than bromo or chloro compounds. In many of the preparations to be described other halogen fatty acids are used. With chloroacetic acid or α-bromopropionic acid on the one hand and glycine or alanine on the other, the following series of compounds has been made.



$$N(CH_2COOH)_3;\ N\begin{matrix}(CH_2COOH)_2\\(CHCH_3COOH)\end{matrix};\ N\begin{matrix}(CH_2COOH)\\(CHCH_3COOH)_2\end{matrix};\ N(CHCH_3\cdot COOH)_3$$

Of these the tricarboxymethylamine is quite outstanding, differing from the others in its insolubility and the ease with which it crystallizes. It also forms an insoluble, easily crystallizable barium salt. All the others are extremely water-soluble as are also their barium salts; so more round about methods had to be used for their separation. An attempt was made to use this characteristic of tricarboxymethylamine to determine quantitatively the glycine content of proteins. Gelatin or silk was hydrolyzed with acid, the hydrolysate made alkaline, and treated with excess of chloroacetic acid and acidified to the turning point of Congo paper with HCl; the tricarboxymethylamine derived from glycine alone is precipitated, in the course of several days, with a yield, amounting to about 75 per cent of the theoretical yield calculated on the glycine content of the protein.

On comparing the relative rates of reaction of iodoacetic acid with —SH and with —NH_2 groups the one with —SH seems to be the more rapid. In the case of cysteine for example, which contains both —SH and —NH_2 groups, it is possible to obtain the S-ether compound with the —NH_2 group unaffected provided too large an excess of iodoacetic acid is avoided. In all cases investigated the reaction with —NH_2 groups is very rapid when carried out under the conditions established for purposes of preparation of the products. At about 80° and in quite alkaline solution the reactions appear to be complete in 15 minutes. However, all these reactions also occur at room temperature and many of the preparations have actually been carried out in this way. The reaction also occurs slowly in the physiological pH range as was determined for the case of glycine. A glycine solution made just alkaline to phenolphthalein, when mixed with an exactly neutralized solution of iodoacetic acid, liberated at room temperature hydriodic acid as was determined by observing the progressive decrease in standard acid required to bring a sample of the mixture to the turning point of methyl red and also by the progressive appearance of iodide ion as determined by silver nitrate. A control solution containing no glycine did not show this effect, so it could not have been due merely to the hydrolysis of iodoacetic acid. This liberation of hydriodic acid continued even after the mixture, as a result of the reaction, had become acid to phenolphthalein. Another experiment in which a solution of glycine and iodoacetic acid was neutralized with excess sodium bicarbonate in such a way that the mixture was always in the $NaHCO_3$-H_2CO_3 buffer range (acid to phenolphthalein) showed that the reaction had reached half completion in about 8 hours at 30°.

Some experiments were also run to determine the relative rates of reaction of iodoacetic acid with the —SH groups of different compounds, cysteine, thioglycolic acid, and thioglycolic acid anilide being chosen. The rates were followed by noting the decrease in iodine consumption of samples of the mixture after various time intervals. Enough sodium carbonate was added in each case to be just equivalent to the initial acids present. The reaction was 70 per cent complete in the case of cysteine in about 10 minutes, with thioglycolic acid anilide in about 30 minutes, and with thioglycolic acid in about 3 hours. This latter example shows that not all —SH compounds react with the same ease as cysteine. Glutathione belongs to the very rapidly reacting —SH compounds.

With these reactions of iodoacetic acid, shown to occur under conditions approaching the physiological, the interpretation of results obtained with this acid will have to be made cautiously. One may say, if the effect of some agent such as an enzyme is destroyed by iodoacetic acid at a pH of 7 to 8, the point of attack of this acid may be considered as a —SH group, provided this is confirmed by some other evidence. If the effect of iodoacetic acid occurs only at pH $>$ 7 or 8, the point of attack may be just as well an amino group. The reaction of iodoacetic acid on hydroxyl groups is probably negligible under conditions of physiological experiments.

EXPERIMENTAL

Iodoacetic acid was prepared from chloroacetic acid and potassium iodide, and recrystallized three or four times from carbon tetrachloride. It was perfectly white and free from free iodine.

Tricarboxymethylamine—Although this compound has already been described by Heintz, the preparation which has been worked out is so much simpler that it seems worth presenting. 1.5 gm. of glycine and 7.5 gm. of iodoacetic acid are dissolved in 20 cc. of water and 16 cc. of 6 M KOH are added. The mixture is warmed in a water bath at 80° and then cooled. HCl is added cautiously until the mixture is just acid to Congo red paper. The product crystallizes out, on scratching a little, as large plates and after drying weighs 3.1 gm. It can easily be recrystallized from hot water. 2.5 gm. are obtained or 66 per cent of the theoretical.

$N(CH_2COOH)_3$. Calculated, N 7.33; found, N 7.42

The crystals melt with decomposition at 230–235°. Fig. 1 shows a potentiometric titration of an aqueous solution of this acid at the glass electrode. 3 equivalents of NaOH are used up; 2

within the acid range. The pK values of these two overlapping steps are somewhere around 3. The third constant is about 10 and should be interpreted as the acidic constant (in Brönsted's sense) of the amino group.

The rest of the carboxymethyl- and carboxyethylamines which were prepared could not be isolated directly because of their great solubility. They are formed by warming together 40 mM of either glycine or alanine and 80 mM of chloroacetic or α-bromopropionic acid with enough potassium hydroxide to neutralize the original acids as well as the halogen acid which splits out as a result of the

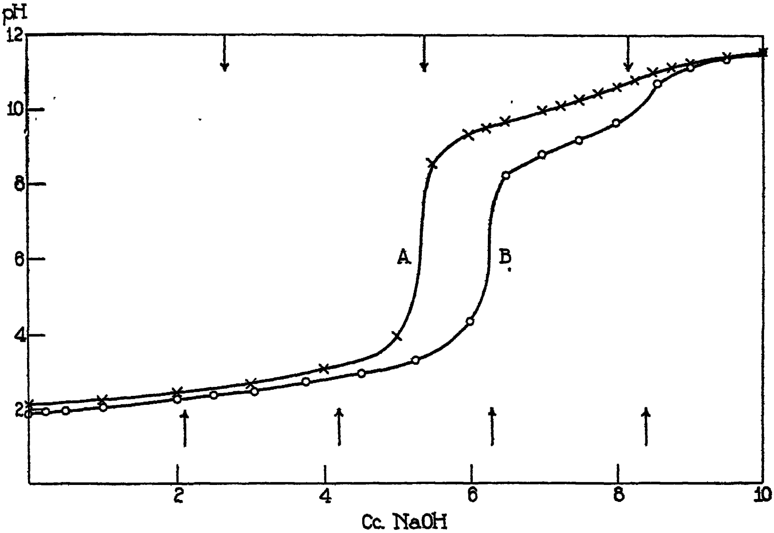
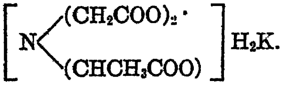
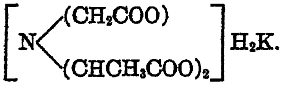


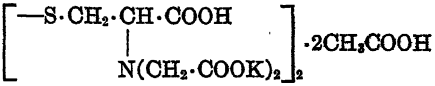
FIG. 1. Curve A, tri(carboxymethyl)amine, 0.0525 gm., titrated with 0.0993 M NaOH. The arrows at the top of the figure show where the 3 equivalents of alkali are used up, as read from the graph, the abscissa of the big jump being taken to calculate the other two steps. Curve B, dicarboxymethylcysteic acid, 0.0647 gm., titrated with 0.0993 M NaOH. The arrows at the bottom of the figure correspond to the successive consumption of 4 equivalents of alkali as deduced from the jump in the curve at the 3rd. These curves were run with a glass electrode, the E.M.F. values being translated to pH by comparison with the E.M.F. read in standard acetate buffer. In each case the end-point as read from the graph is between 3 and 4 per cent short of the end-point as calculated from the weight of substance and the formula deduced from analysis of the compound.

reaction. These mixtures are all warmed at about 80° for half an hour, 6 M HCl is added until the solution is acid to Congo red paper, and then it is evaporated *in vacuo* to dryness. The residue is extracted with about 50 cc. of hot glacial acetic acid containing about 4 gm. of potassium acetate and the hot acetic acid filtered off quickly with suction. After cooling the filtrate it may be necessary to filter again if potassium bromide separates. About 400 to 500 cc. of absolute alcohol are then stirred into the filtrate and the mixture set on ice. In the case of the two unsymmetrical amines precipitation is immediate but the products are not crystalline, while with the two symmetrical amines complete separation may take 4 days to a week but the products are crystalline. After filtering off the deposits, washing with absolute alcohol, and drying *in vacuo*, these analytical results were obtained.

 $\left[N\begin{matrix}\diagup(CH_2COO)_2\\ \diagdown(CHCH_3COO)\end{matrix}\right]H_2K.$	Calculated.	N 5.76,	K 16.04
	Found.	" 5.48,	" 14.59
 $\left[N\begin{matrix}\diagup(CH_2COO)\\ \diagdown(CHCH_3COO)_2\end{matrix}\right]H_2K.$	Calculated.	" 5.45,	" 15.17
	Found.	" 5.69,	" 15.18
$[N\ (CHCH_3COO)_3]H_2K.$	Calculated.	" 5.17,	" 14.39
	Found.	" 5.47,	" 14.88

The first two of these compounds are extremely hygroscopic and difficult to obtain in a form suitable for analysis.

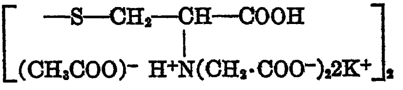
Tetrapotassium Tetracarboxymethylcystine Diacetate—A solution of 10.7 gm. of cystine in 20 cc. of water and 13.7 cc. of 6.4 M KOH is mixed with one of 51.8 gm. of iodoacetic acid in 20 cc. of water and 43 cc. of 6.4 M KOH. To the mixture 35 cc. more 6.4 M KOH are added and the clear solution either heated in a water bath at 90° for 30 minutes or allowed to stand at room temperature for an hour or two, after which it is filtered if necessary. To the clear solution 750 cc. of alcohol are added and the mixture set on ice for 2 hours. A heavy liquid separates at the bottom of the beaker. To obtain an analyzable pure substance from the liquid, the following procedure was adopted. The main body of liquid is decanted and the heavy liquid residue is washed by decantation with three 25 cc. portions of alcohol and two 25 cc. portions of acetone. The remaining acetone is then removed by gentle warming. On cooling, the residue often crystallizes at this point. It is dissolved in 150 cc. of glacial acetic acid containing in solution 18 to 20 gm. of potassium acetate. When it is completely dissolved, which process requires considerable stirring, the solution must be perfectly clear; if it is not, it is filtered. 600 cc. of absolute alcohol are added, producing a heavy white precipitate. The mixture is now set in a water bath at 70° for an hour and then cooled. The precipitate becomes somewhat sandy though not definitely crystalline. It is filtered off and washed with a large volume of absolute alcohol and then dried over H_2SO_4 in a vacuum. 33.0 gm. of product are obtained, a yield of 88 per cent. The formula appears to be



$$\left[\begin{array}{l}\text{—S·CH}_2\text{·CH·COOH}\\ \qquad\qquad\quad |\\ \qquad\quad\text{N(CH}_2\text{·COOK)}_2\end{array}\right]_2\cdot 2\text{CH}_3\text{COOH}$$

Calculated. S 8.60, N 3.76, K 20.98
Found. " 8.34, " 3.84, " 21.55

The product is perfectly white and is extraordinarily easily soluble in water. This property may make it of some interest, being an easily obtainable derivative of cystine which is soluble in both the disulfide and the sulfhydryl forms. The aqueous solution is acid in reaction. As to the formulation of this compound, one way is, as shown above, to regard the acetic acid as analogous to the water of hydrates. Another way is to regard the compound as an amphi-salt in the sense described by Pfeiffer (4), that is



$$\left[\begin{array}{l}\qquad\text{—S—CH}_2\text{—CH—COOH}\\ \qquad\qquad\qquad\quad |\\ \text{(CH}_3\text{COO)}^-\ \text{H}^+\text{N(CH}_2\text{·COO}^-)_2\text{2K}^+\end{array}\right]_2$$

No decision between these formulations being yet possible, it suffices to classify this compound in the group of crystalline compounds consisting of a molecule of an ampholyte plus 1 molecule of a neutral salt.

Tetracarboxymethylcystine—Attempts to prepare the free acid itself were largely unsuccessful. The barium salt was precipitated as an amorphous solid from a slightly alkaline solution of the potassium salt just described. This barium salt is filtered off, washed well with water, resuspended in water, and sulfuric acid added in the cold until there is just a very slight excess of the acid. The barium sulfate is filtered off, the clear solution evaporated *in vacuo* to dryness, and the residue ground up with acetone. The white sandy mass that is left is filtered off, washed with acetone, and dried. Most preparations gave values very low in sulfur, indicating some kind of decomposition. Only one preparation gave analytical figures which began to appear reasonable.

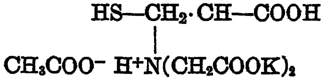
$[-S\cdot CH_2\cdot CHN(CH_2COOH)_2\cdot COOH]_2$.	Calculated.	S 13.55, N 5.93
	Found.	" 14.40, " 5.98

Dicarboxymethylcysteic Acid—This is the only compound in this series which could be obtained in a distinctly crystalline condition. The potassium salt described above is dissolved in a little water, bromine added to a slight excess, the solution made alkaline, and the barium salt of the sulfonic acid is precipitated. This is washed with much water, decomposed with exactly an equivalent amount of sulfuric acid, and the filtered solution evaporated to dryness *in vacuo*. The residue is ground up with acetone, filtered, and dried. It is then dissolved in a small quantity of absolute methyl alcohol, filtered, and about 10 volumes of acetone added. After standing on ice a few days a crystalline deposit of short needles separates. This is filtered off, washed with acetone, and dried.

$HO_3S\cdot CH_2\cdot CHN(CH_2COOH)_2\cdot COOH\cdot H_2O$.	Calculated.	S 10.55, N 4.62
	Found.	" 10.36, " 4.47

An acid titration curve for this substance is given in Fig. 1. This shows three strong acid constants ($pK < 3$) and a fourth in the neighborhood of 9.5. According to expectation, instead of the 3 equivalents of alkali used up in the case of the glycine compound plotted in Fig. 1, 4 equivalents are used up here due to the additional presence of a SO_3H group.

Dipotassium Dicarboxymethylcysteine Monoacetate—Attempts to prepare the corresponding —SH compound also failed to yield a crystalline product. By reduction of the above potassium compound with tin in 4 M HCl, neutralization of most of the acid, removal of tin with H_2S, and evaporation of the resulting solution to dryness *in vacuo*, an oily product is obtained which is extracted with a mixture of 50 cc. of glacial acetic acid containing about 5 gm. of potassium acetate. The acetic acid is filtered off and the product precipitated with 4 volumes of absolute alcohol. The mixture is digested at 80° for an hour, which renders the precipitate somewhat sandy but not definitely crystalline. It is filtered off and washed with absolute alcohol.



$$\begin{array}{l} HS\text{—}CH_2\cdot \underset{|}{C}H\text{—}COOH \\ CH_3COO^- \; H^+N(CH_2COOK)_2 \end{array}$$

Calculated. S 8.58, N 3.75, K 20.90
Found. " 10.06, " 3.99, " 21.83

Titration of this compound with alcoholic iodine gave an equivalent weight of 338 instead of the theoretical 373, but the end-point is very unsatisfactory. That point is taken at which the iodine color suffuses the whole solution for a few seconds, but much more iodine may be added that is very slowly consumed probably causing further oxidations such as investigated by Simonsen (5). There can be no doubt that the expected substance has been obtained, although not in a perfectly pure state owing to the lack of a well crystallized salt. In alkaline solution it gives oxidized complex compounds with cobalt just as cysteine itself does (6). Of interest also is the fact that it does not give the Sullivan reaction characteristic of cysteine.

In addition to these aliphatic amines the action of iodoacetic acid on aromatic amines has also been tried. The neutralized iodoacetic acid and *p*-phenylenediamine are warmed together with enough alkali to neutralize the HI which splits out. The reaction is very rapid, 15 minutes being enough for completion. Both the barium salt and the free acid are well crystallized compounds.

Tetracarboxymethyl-p-Phenylenediamine—This compound melts with decomposition at 165°. On partial oxidation with bromine water it develops a semiquinoid violet dyestuff analogous to Wurster's blue, showing two distinct absorption bands, whose wavelengths are somewhat different from all Wurster's dyes known to us and may be described at another occasion (*cf.* (7)).

$(HOOC\cdot CH_2)_2\cdot N\cdot C_6H_4\cdot N(CH_2\cdot COOH)_2$. Calculated. N 8.24
Found. " 8.48, 8.40

Tetracarboxymethyl-p-Phenylenediamine Barium Salt—

$Ba_2(OOC\cdot CH_2)_4N_2\cdot C_6H_4$. Calculated. N 4.59, Ba 45.04
Found. " 4.38, " 45.25

Some products of the interaction of iodoacetic acid on —SH compounds had already been prepared when the work of Dickens appeared, describing those of cysteine and of glutathione. As the compound with cysteine we have prepared was made by a much simpler method, has been recrystallized, and gives a much higher melting point than the one reported by Dickens, it seems advisable to describe its preparation.

5.5 gm. of cysteine hydrochloride and 3.6 gm. of chloroacetic acid are dissolved in 10 cc. of water, and 17 cc. of 6.7 M KOH are added. The heat of neutralization of the acids and the heat of reaction make the mixture quite hot. After 5 minutes it should still be alkaline to litmus. Add glacial acetic acid until the solution is just acid to litmus and let stand on ice 2 hours. Filter off the cystine. 6 M HCl is now added so the mixture becomes just blue to Congo red paper, and it is again set on ice for 2 hours. The crystalline precipitate which has separated is filtered off, dissolved in 200 cc. of boiling water, and the hot solution filtered and set on ice. 6-Sided plates separate. They differ from cystine in not being regular hexagons as well as their easy solubility in hot water. 70 per cent of the theoretical yield is obtained after one recrystallization.

$HOOC \cdot CHNH_2 \cdot CH_2 \cdot S \cdot CH_2COOH$.

Calculated.	N 7.82,	S 17.88,	C 33.51,	H 5.06
Found.	" 7.75,	" 17.81,	" 32.97,	" 5.24

The melting point is 175–176° with decomposition.

The corresponding compound with thioglycolic acid anilide is made similarly by warming together in solution equivalent quantities of the anilide and of iodoacetic acid neutralized with Na_2CO_3. After 20 minutes the mixture is cooled, acidified with HCl, and set on ice. The crystals may require some scratching before appearing. They can be recrystallized from a small volume of hot alcohol. After drying *in vacuo* 80 per cent yields can easily be obtained. The corrected melting point is 99–100°.

$C_6H_5 \cdot NH \cdot CO \cdot CH_2 \cdot S \cdot CH_2 \cdot COOH$.

Calculated.	S 14.21,	N 6.22,	C 53.38,	H 4.93
Found.	" 14.30,	" 5.77,	" 53.40,	" 5.11

The glutathione compound was prepared by allowing a solution of crystallized glutathione and iodoacetic acid just neutralized with Na_2CO_3 to stand in a heavy veronal buffer at room temperature. The precipitated veronal is removed by filtration, carbonate is removed with barium, and the barium salt of the glutathione compound precipitated with alcohol. This is filtered, washed, dissolved in water, and the lead salt precipitated. From this the desired compound is obtained by treatment with H_2S, filtration, and evaporation to dryness.

$C_{10}H_{16}O_6N_3S \cdot CH_2COOH \cdot H_2O$.

Calculated.	S 8.35,	N 10.96,	C 37.60,	H 5.53
Found.	" 8.30,	" 10.73,	" 35.10,	" 5.34

Thiodiglycolic acid has also been isolated from the reaction of iodoacetic acid and thioglycolic acid.

SUMMARY

Iodoacetic acid reacts not only with sulfhydryl compounds but also with amino compounds both aliphatic and aromatic, particularly with amino acids. All H atoms of the —SH or —NH_2 group are easily substituted by the radical —CH_2COOH. For both these groups of reactions examples are described. The following compounds have been prepared: tri(carboxymethyl)amine,[1] di-(carboxymethyl) (α-carboxyethyl)amine potassium salt, carboxymethyldi(α-carboxyethyl)amine potassium salt, tri(α-carboxyethyl)amine potassium salt, tetracarboxymethylcystine, dicarboxymethylcysteic acid, dicarboxymethylcysteine acetate, tetracarboxymethyl-*p*-phenylenediamine, S-carboxymethylcysteine,[1] S-carboxymethylglutathione,[1] S-carboxymethylthioglycolic acid anilide.

BIBLIOGRAPHY

1. Lundsgaard, E., *Biochem. Z.*, **217**, 162 (1930); **220**, 1 (1930).
2. Dickens, F., *Biochem. J.*, **27**, 1141 (1933).
3. Heintz, W., *Ann. Chem.*, **122**, 257 (1862); **145**, 49 (1868).
4. Pfeiffer, P., Organische Molekülverbindungen, Stuttgart, 139 (1927).
5. Simonsen, D. G., *J. Biol. Chem.*, **101**, 35 (1933).
6. Schubert, M. P., *J. Am. Chem. Soc.*, **53**, 3851 (1931).
7. Michaelis, L., *J. Am. Chem. Soc.*, **53**, 2953 (1931).

---

[1] These compounds had been prepared by different methods previously.

THE RÔLE OF COPPER IN CARBOHYDRATE METABOLISM

BY H. L. KEIL AND VICTOR E. NELSON

(*From the Laboratories of Physiological Chemistry, Iowa State College, Ames*)

(Received for publication, May 11, 1934)

Copper has been designated as one of the dietary essentials by a number of investigators and appears to occupy a unique position as a necessary supplement to iron for the synthesis of hemoglobin within the animal body. However, our present knowledge concerning the function of copper, other than as a hematopoietic agent, is limited. In view of the fact that this element is found by analysis in many organs, one is led to believe that its presence is not accidental, but perhaps is required to promote normal activity in that portion of the body containing it. Any attempt to disclose specific organic effects on living rats receiving copper would be difficult. It therefore occurred to us that an investigation of some major physiological process might yield information indicative of another rôle of copper in nutrition. With this idea in mind a series of glucose metabolism experiments was initiated.

Gettler and Lindeman (1) have reported abnormally high blood sugar in pernicious anemia. They interpret the results to mean either a failure in glucose oxidation or a disturbance of the glycogen-glucose equilibrium. Glucose tolerance tests performed by Rennie (2) on patients with pernicious anemia, after a 10 to 12 hour fasting period, showed higher values than were obtained on the controls. No correlation could be drawn between hemoglobin and blood sugar levels. The work presented in this paper consists primarily of a comparison of glucose utilization by anemic rats, before and after copper and iron supplementation, with that of normal animals. Sugar tolerance tests have been selected as a means of determining the rate of glucose metabolism in an attempt to answer the following questions: (1) Does copper influence other physiological processes aside from that of hemoglobin formation? (2) Can the rate of disappearance of glucose from the blood stream be correlated with hemoglobin content in nutritional anemia? (3) Is the fasting blood sugar level higher in anemic than in normal animals?

EXPERIMENTAL

Anemic rats maintained upon a milk ration low in copper served as test animals because hemoglobin and stored copper could be markedly reduced simultaneously. The blood sugar changes in these animals were then studied first from the standpoint of anemia and next after copper administration. The experiments to be described comprise 78 anemic and eighteen normal rats equally divided with respect to sex. Six animals of the same sex were housed together in a galvanized iron wire cage resting on glass rods over a pan containing wood shavings. All animals were selected from stock colony mothers at 30 days of age. Those to become anemic were fed upon whole milk—from pure-bred Holstein cows—collected in glass containers, in order to eliminate copper and iron contamination. A copper and iron depletion period of from 8 to 10 weeks was prescribed for the anemic rats, while the normal controls were fed a stock diet for the same length of time. Hemoglobin determinations were made periodically by the Newcomer acid hematin method.

After the hemoglobin had fallen to a sufficiently low level the experiments were started by fasting the rats for 20 hours previous to bleeding. Preliminary tests served to eliminate those showing either signs of nervousness during the tail bleeding operation or an unwillingness to drink readily from a pipette. Only rats demonstrating consistent glucose values were continued on experiment, since it is well known that fear and emotion will cause fluctuations in blood sugar determinations. The regular procedure was to determine first the blood sugar level after a 20 hour fast. Each animal was then given orally a pure glucose solution in an amount affording exactly 200 mg. of glucose per 100 gm. of body weight. Individual blood analyses were carried out at ½, 1, and 2 hours after glucose ingestion. Previous experience had shown 10 minute intervals to be very annoying to the unanesthetized animal; besides, the blood sugar curve rose steadily during the first 30 minutes after which it declined at a similar rate.

Blood sugar determinations were made by the method of Folin and Malmros (3). A slight modification was introduced because of the difficulty encountered in obtaining the 0.1 cc. of blood, required by the Folin pipette, from the tail of the rat. Newcomer hemoglobin pipettes proved much better for our method of sampling. Two 20 c.mm. volumes of blood from an individual were accurately measured and delivered into a 15 cc. centrifuge tube containing 4 cc. of Folin's dilute tungstic acid solution. After thoroughly mixing and centrifuging, the supernatant liquid was poured into a Pyrex tube marked at a 25 cc. volume content. This protein-free filtrate represented 40 c.mm. of blood and equaled the amount taken for a single determination in the Folin and Malmros method. Their procedure was followed from this point. Table I offers a comparison of glucose values obtained with the Folin and also the Newcomer pipette from the same blood sample. The blood was collected from normal rats after a 9 hour fast. They were stunned and immediately bled into beakers containing a little sodium oxalate to prevent clotting.

TABLE I

Effect of Sampling on Accuracy of Method

Rat No.	Glucose per 100 cc. blood	
	Folin pipette	Newcomer pipette
	mg.	*mg.*
1	113	109
2	102	101
3	111	111
4	114	112
5	103	102
6	107	104
7	96	95
8	89	90
Average..............	104.3	103.0

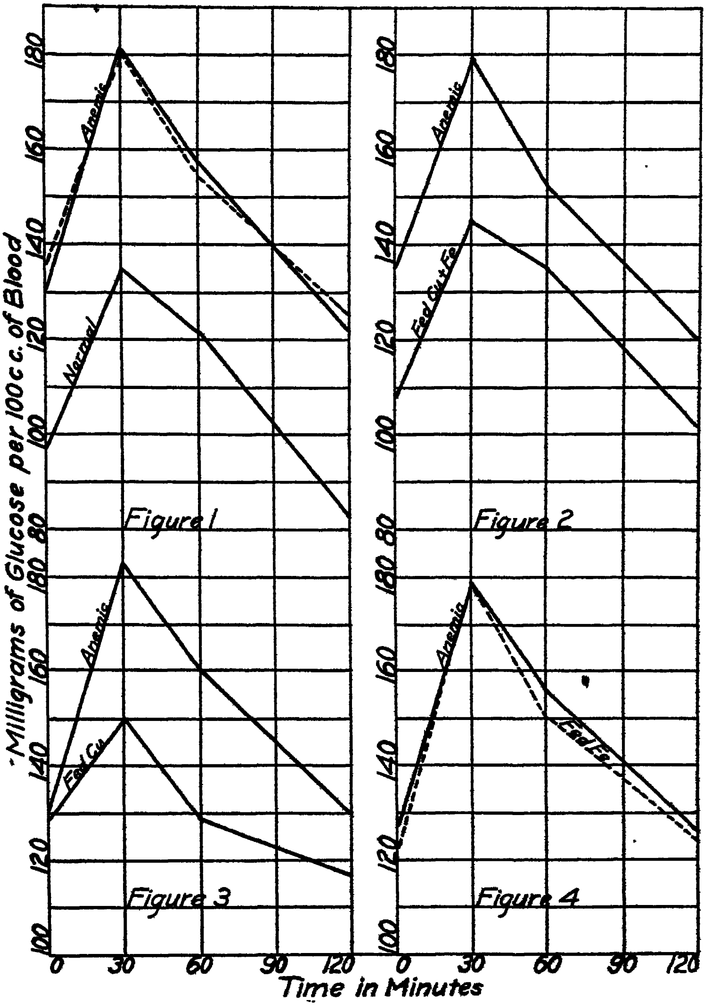
Copper as $CuSO_4 \cdot 5H_2O$ was fed orally at a level of 0.1 mg. daily in the milk. The copper salt was made by dissolving a Hilger spectrograph electrode in dilute HNO_3; the resulting $Cu(NO_3)_2$ was filtered, converted to $CuSO_4$ by digesting with H_2SO_4, and finally crystallized from a dilute solution. Selected crystals dissolved in copper-free water formed the solution which was fed.

Iron as $FeCl_3$ was administered at a rate of 1.0 mg. daily in the milk. Pure $FeCl_2$ was made by dissolving Baker and Adamson's standardization iron wire in a 1:1 HCl solution. The filtered crystals were placed in a solution of 2 per cent HCl and freed of copper by bubbling H_2S through the liquid for 30 minutes; after filtration, the iron was oxidized to $FeCl_3$ by boiling with HNO_3 and was converted to $Fe(OH)_3$ upon the addition of NH_4OH. Ferric hydroxide, thus obtained, was washed well on a Buchner filter and put into solution as $FeCl_3$ by bubbling HCl through the suspension. The solutions as fed were analyzed for iron by the KCNS method to insure quantitative administration. Both $CuSO_4$ and $FeCl_3$ solutions, made in the manner described, proved to be free from contamination of each other. They were tested separately on anemic rats and found to produce no hematopoietic response.

DISCUSSION

The blood sugar tolerance curves shown in Figs. 1 to 4 represent composite data obtained from a number of rats grouped according to individual hemoglobin values at the beginning of the experiment. Fig. 1 depicts the performance of forty-eight anemic and eighteen normal animals. Average hemoglobin values for the anemic males and females were 4.8 and 5.1 per cent respectively. It can be seen that a high fasting level and peak exist in nutritional anemia, although the rate of return to initial conditions is practically the same as in normal controls having a hemoglobin content of 15 per cent. Intestinal absorption is not impaired in anemia as evidenced by the uniform time of maximum rise in blood sugar. Sugar metabolism appears to go on at the same rate in both sexes, due to the fact that ovulation does not occur in anemic rats.

Six males were tested (Fig. 2) first at a hemoglobin level of 4.1 per cent as shown in the upper curve. They were then fed 0.1 mg. of copper together with 1.0 mg. of iron daily in the milk for 15 days, when their hemoglobin values averaged 11.55 per cent; at this point glucose tolerance was determined. Results of this experiment make up the lower curve (Fig. 2). That the glucose utilization approaches a normal state is manifested by both a lower fasting level and peak. A comparison of the following curves—Fig. 2 (upper), Fig. 2 (lower), and Fig. 1 (lower) with corresponding hemoglobin values of 4.1, 11.55, and 15.0 per cent—establishes a corre-



FIGS. 1 to 4. Effect of anemia, copper, and iron on blood sugar level and glucose tolerance of rats. In Fig. 1, solid upper curve is composite for twenty-four anemic males; upper dotted curve, composite for twenty-four anemic females; lower curve, data on eighteen normal animals receiving stock ration. Fig. 2 shows the results obtained on six males before and after feeding 0.1 mg. of Cu + 10 mg. of Fe for 15 days; Fig. 3, for six males and six females before and after a 10 day Cu feeding period; Fig. 4, for six males and six females before and after feeding 1.0 mg. of Fe for 10 days.

lation between amount of hemoglobin and reducing power of the blood.

The top curve (Fig. 3) was derived from data obtained on six male and six female rats, whose hemoglobin values averaged 5.4 per cent. After a 10 day copper feeding period their glucose tolerance changed markedly as is shown by the lower curve (Fig. 3). It can be clearly seen that copper ingestion has lowered the maximum point by some 30 mg. while the fasting level remained practically stationary. Copper administration did not cause regeneration of hemoglobin in these animals; therefore, the effect must have been due to this element alone. Furthermore, the fasting level has been shown to remain high as long as anemia persists (Figs. 1 and 2). Other data, not included in this paper, substantiate these results.

We have no direct experimental evidence as to the mode in which copper acts to reduce the glucose peak in anemic rats. The most logical explanation seems to involve an improvement in liver function to bring about an acceleration of glycogen formation and thus a rapid removal of glucose from the blood. Unpublished data, obtained in this laboratory from anemic rat urine, show no impairment of pancreatic activity. The possibility of a direct oxidation catalyzed by copper either in the blood stream or in muscular tissue is overruled, provided that glucose is assumed to be the only reducing agent in anemic blood. And if this assumption is correct, then a lowered fasting value should result from copper administration. Pure iron alone does not affect glucose tolerance (Fig. 4). The uppermost curve produced from data on twelve anemic rats divided equally as to sex falls almost upon the lower curve obtained from the same rats after 1.0 mg. of iron had been fed daily for 10 days. Hemoglobin values averaged 6.2 per cent and were not altered by iron feeding.

SUMMARY

1. Oral administration of copper alone to anemic rats produces a different type of glucose tolerance curve than that obtained from the same animal before the mineral ingestion. A significant lowering of the maximum point demonstrates a rôle of copper in nutrition aside from hemoglobin formation. The fact that hemoglobin values were unaltered proves that the effect was due not to ordinary physiological oxidative processes, but must have arisen from a hitherto undescribed property of copper.

2. Pure iron alone does not improve glucose utilization as shown by sugar tolerance tests.

3. Any increase of hemoglobin in anemic rats produces a proportional increase in sugar tolerance coupled with a lowered glucose level in the blood after a 24 hour fast.

4. Blood sugar values determined after 20 hours of fasting are consistently higher in animals suffering with nutritional anemia as compared to normal controls of the same age; whether or not this reducing substance is all glucose has not been determined. Both sexes perform similarly on glucose tolerance experiments when the hemoglobin is at an anemic level.

BIBLIOGRAPHY

1. Gettler, A. O., and Lindeman, E., *Arch. Int. Med.*, **26**, 453 (1920).
2. Rennie, T. A. C., *J. Lab. and Clin. Med.*, **16**, 557 (1931).
3. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 115 (1929).

THE USE OF THE HAGEDORN-JENSEN METHOD IN THE DETERMINATION OF SKIN GLUCOSE*

BY DONALD M. PILLSBURY AND GEORGE V. KULCHAR

(From the Department of Dermatology and Syphilology and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

(Received for publication, March 15, 1934)

A study of the relation of various skin disorders to disturbances of carbohydrate metabolism has led us to a consideration of the various methods of glucose determination in skin. A micromethod enabling the use of small samples seemed preferable since large specimens of human skin are often difficult to obtain. Urbach and Fantl (1) have employed a direct adaptation of the Hagedorn-Jensen blood method for the determination of skin glucose. Samples of skin which have been sliced and teased apart are extracted and precipitated in the regular Hagedorn-Jensen zinc sulfate-sodium hydroxide mixture. Following filtration and washing, the reducing substances of the filtrate are determined by the ferricyanide reagent.

The paper of Urbach and Fantl contains no indication that the method had been studied critically. There are several possible sources of error which we have thought warranted study: the length of time of extraction of the skin, the possibility of glycogenolysis during the extraction of the tissue in the hot alkaline zinc sulfate-sodium hydroxide mixture, and the possibility of enzymatic glucose formation or destruction in skin after removal from the animal. We have compared the skin glucose values obtained by the Hagedorn-Jensen method with values obtained by the Somogyi-Shaffer-Hartmann technique.

Method

Both guinea pigs and rabbits, chiefly the latter, were used. The animals were fasted for periods varying from 24 to 72 hours. The

---

* This research was aided by a grant from the Special Research Fund of the University of Pennsylvania.

skin over the mid-portion of the abdomen was shaved 48 hours before removing the skin samples, irritation being avoided as much as possible. Skin from this region was used because of the ease with which it could be dissected from the subcutaneous tissue.

Sodium amytal (75 mg. per kilo), given intraperitoneally, was used for anesthesia. Following administration of the drug the animal was allowed to remain quietly in its cage until relaxation had occurred. The sample of skin was quickly removed, cut into very thin slices with scissors, and portions totaling 100 to 150 mg. were dropped into weighed corked tubes containing 1 cc. of 0.1 N sodium hydroxide and 5 cc. of 0.45 per cent zinc sulfate. The tubes were then weighed to 0.1 mg. and placed in a boiling water bath for 3 minutes. The elapsed time between removal of the skin from the animal and the beginning of extraction seldom exceeded 4 minutes.

On removal from the water bath the contents of the tubes were filtered through washed cotton-wool which had been rather tightly packed in the funnel tube. The precipitate was washed three times with 3 cc. portions of hot distilled water. The reducing substances were then determined by the Hagedorn-Jensen method as modified by Cole (2).

To determine the effect of longer extraction and varying alkalinity of the extracting medium this procedure was varied by extracting the tissue for periods up to 120 minutes in the regular zinc sulfate-sodium hydroxide mixture, in water, in 0.45 per cent zinc sulfate, and in 0.1 N sodium hydroxide. The results obtained by varying the time of extraction of skin in the hot $ZnSO_4$-NaOH mixture are recorded in Table I. It may be seen the reducing substances in solution increase with the extraction time until 1 hour is reached, when no further rise is noted. A similar rise in the reducing values was obtained when water, 0.45 per cent $ZnSO_4$, or 0.1 N NaOH was used as the extracting medium. It appeared from these latter experiments that the alkalinity of the $ZnSO_4$-NaOH mixture was not responsible for the rise in reducing values with increased extraction time. Experiments were performed in which 10 mg. of glycogen were added to each of a duplicate series of tubes and the effect of increased extraction time noted. The addition of glycogen did not affect the results.

Determination of the non-fermentable reducing substances was made by adding 1 cc. of a 10 per cent yeast suspension, prepared after the method of Benedict (3), to the filtrate of skin which had been extracted in distilled water for varying periods. The mixture was incubated at 37° for 15 minutes, and zinc sulfate and sodium hydroxide added in amounts sufficient to bring the final solution to the proper concentration. As the yeast blank determination is large, care must be taken to use uniform samples of the yeast suspension for fermentation and for the blanks.

TABLE I

Relation of Time of Extraction to Reducing Substances Extracted, in Rabbits

All extractions are made in $ZnSO_4$-NaOH at 100°; the results are given in mg. of glucose per 100 gm. of tissue.

Experiment No.	3 min.	15 min.	30 min.	60 min.	120 min.
XIII	194		267		
XVI	125		211		
XVII	137		204		
XVIII	137		174		
XXIII	124	171			
XXIV	125	165	219		
XXVI	112		179		
XXVII	164	192	200	223	223
XXX, guinea pig	143		250*	226	240
XXXI	144			223	233
XXXV	131			199	

* Extracted 45 minutes.

The total reducing substances and the non-fermentable reducing fraction were determined in samples of skin extracted for periods varying from 3 minutes to 2 hours. The data are given in Table II. It will be seen that no non-fermentable reducing substances were found in the filtrates of tissue extracted for 3 minutes and that the rise in total reducing substances occurring with increased extraction time seemed to be due to the increased amounts of non-fermentable reducing substances extracted. In this connection it is interesting to note that some investigators of tissue glucose have employed extraction times of 1 hour or more (Bischoff and Long (4); Folin, Trimble, and Newman (5)). Our results would indicate that in finely sliced skin long extraction times are not necessary for the recovery of glucose and are not desirable on account of the extraction of non-fermentable reducing substances.

With the optimum time of extraction established, recovery of added glucose was studied. Three experiments were performed as follows: seven sliced skin samples approximately 100 mg. in weight were taken from the same animal and weighed in stoppered test-tubes to 0.1 mg. To each of four of the tubes was added 0.2 mg. of glucose in 1 cc. of water. The tubes were thoroughly shaken and the reducing substances determined by extracting for 3 minutes with the ZnSO$_4$-NaOH mixture at 100°. The average of the three blank determinations was used for calculating recovery of added glucose. The accompanying tabulation shows the individual blank determinations and the percentage recoveries of added glucose.

TABLE II

Increase in Non-Glucose Reducing Materials with Increase in Time of Extraction

Experiment No.	Time of extraction	Glucose per 100 gm. tissue		
		Total reducing material	Non-fermentable reducing material	Glucose (calculated)
	min.	*mg.*	*mg.*	*mg.*
XXXII	3	126	0	126
	30	165	46	119
	60	178	Lost.	
	90	201	62	139
	120	218	100	118
XXXV	3	131	0	131
	60	199	66	133
XXXVI	3	161	0	161
XXXI	3	144	0	144
	60	223	78	145
	90	215	80	135

Experiment No.	Skin glucose	Recovery of added glucose
	mg. per cent	*per cent*
I	133, 141, 139, average 137.7	104, 103, 98, 105
II	161, 158, 162, " 160.3	111, 98, 99, 105
III	160, 165, 158, " 161	102, 94, 97, 92

In samples of skin incubated at 37.5° with normal saline and 2 per cent $NaHCO_3$ solution, significant changes in the reducing values were not observed in tissues incubated less than 1 hour. From these experiments we may conclude that slight delay in the preparation of skin samples does not result in increased reducing values. Incubation for 2 hours resulted in slightly increased glucose values (increases of 20 to 40 mg. per cent). Addition of 20 mg. of glycogen to the incubating tube containing 100 mg. of tissue resulted in marked increase of reducing substances after 2 hours (70 to 150 mg. per cent).

TABLE III

*Comparative Determinations of Skin Glucose Made by Hagedorn-Jensen and Shaffer-Hartmann Methods**

Glucose per 100 gm. tissue	
Hagedorn-Jensen	Somogyi-Shaffer-Hartmann
mg.	*mg.*
120	104
113	108
84	63
92	89
111	99

* We are indebted to Mr. L. S. Smelo and Mr. F. M. Kern for the analyses by the Shaffer-Hartmann method.

In two experiments in which samples of skin were frozen in liquid air, ground, and the reducing substances determined after extraction of the ground tissue for 3 minutes in the $ZnSO_4$-NaOH mixture, comparison with the glucose values obtained on sliced skin of the same animal prepared in the usual way revealed higher values in the frozen tissue. The differences were 18 mg. per cent in one experiment and 32 mg. per cent in the other. The higher values from ground tissue which had been frozen may have been due to extraction of non-glucose reducing material.

An effort was made to compare the Hagedorn-Jensen method as used with skin with another of the commonly used procedures for the determination of glucose. We attempted tungstic acid precipitation as employed by Folin, Trimble, and Newman (5) on human and dog skin. Using rabbit skin, we obtained precipitates with this method that could not be removed by filtration in most instances. When sufficient filtrate was obtained for a Shaffer-Hartmann determination, the results were highly variable but were most commonly very low, under 10 mg. per cent of glucose. Somogyi filtrates for comparative determinations by the Shaffer-Hartmann technique were obtained in a manner similar to that employed by Bischoff and Long (4) in glucose determinations on muscle. When filtrates of skin which had been extracted in distilled water for 1 hour were used, rubbery precipitates were often obtained which made filtration at times impossible. A shorter extraction time of 15 minutes gave satisfactory filtrates. Determinations of glucose by the Hagedorn-Jensen method and the Somogyi-Shaffer-Hartmann method on skin from the same animal are recorded in Table III. It will be observed that in all instances the Shaffer-Hartmann values are below the Hagedorn-Jensen values. It is interesting to note that when blood is analyzed by these two procedures the same relations are obtained.

SUMMARY

Determinations of the skin glucose in amytalized rabbits and guinea pigs by the Hagedorn-Jensen technique indicate that (1) extraction of sliced skin for 3 minutes in the $ZnSO_4$-NaOH mixture at 100° is sufficient to extract all of the glucose; (2) no appreciable amount of the non-glucose reducing substances is extracted in this time; (3) increases in reducing materials obtained with increased extraction times are due to non-fermentable reducing substances; and (4) recovery of added glucose is satisfactory. Similar skin glucose values are obtained with the Hagedorn-Jensen and Somogyi-Shaffer-Hartmann procedure.

BIBLIOGRAPHY

1. Urbach, E., and Fantl, P., *Biochem. Z.*, **196**, 471 (1928).
2. Cole, S. W., Practical physiological chemistry, Baltimore, 7th edition, 425 (1926).
3. Benedict, S. R., *J. Biol. Chem.*, **76**, 457 (1928).
4. Bischoff, F., and Long, M. L., *J. Biol. Chem.*, **87**, 47 (1930).
5. Folin, O., Trimble, H. C., and Newman, L. H., *J. Biol. Chem.*, **75**, 263 (1927).

CHANGES IN THE BLOOD CALCIUM AND PHOSPHORUS PARTITION DURING THE LIFE CYCLE OF THE CHICKEN

By V. G. HELLER and HENRY PAUL

(From the Department of Agricultural Chemistry Research, Oklahoma Agricultural Experiment Station, Stillwater)

and R. B. THOMPSON

(From the Poultry Department, Oklahoma Agricultural Experiment Station, Stillwater)

(Received for publication, June 4, 1934)

The inorganic calcium and phosphorus content of the blood serum has been of interest in mineral metabolism studies for many years. With an increased knowledge of the importance of these ions in certain deficiency diseases, numerous investigations have been made of the calcium-phosphorus ratios existing in these conditions. In nutritional disturbances in chickens an even greater problem is presented due to the occurrence of various bone maladies not recognized in mammals and for which no satisfactory explanation has been offered by the calcium-phosphorus balances. It, therefore, becomes necessary to seek further explanation of these conditions. In a previous article from this laboratory (1), it was reported that the phosphorus content of the blood of the fowl is peculiar in that it is much greater than that of mammals and the inorganic phosphorus content of the serum, as usually determined, composes only a small part of the whole. The theory was offered that other fractions possibly contribute to bone formation as well as the inorganic phosphorus. In the case of the calcium content an equally unique condition exists. In mammals this ion changes very little throughout the life cycle but in fowls the calcium increases over 100 per cent at the time of egg production. The fact that the total calcium content consists of several fractions has been reported by Benjamin and Hess (2), by Correll and Hughes (3), and by this department (4). The possibility that changes may occur in some of the fractions which would not be indicated by the usual determinations has suggested a partition study throughout the complete life cycle of the hen.

EXPERIMENTAL

300 white Leghorn chickens of the same strain and age were secured from the college Poultry Department. These were placed in brooders, and from this group two lots of 50 healthy female chickens were selected, placed in pens, and fed an all mash ration composed of cereal grains supplemented with animal and vegetable proteins, vitamins, and adequate amounts of calcium and phosphorus in the postulated correct proportions. Analyses of the rations showed the protein, calcium, and phosphorus percentages to be 17, 1.21, and 0.776, respectively. Records of food consumption, growth curves, and frequent examinations were made so that none but healthy, active birds were kept in the lots.

The blood was drawn in all cases by heart puncture. For phosphorus determinations it was delivered into chilled oxalate-coated flasks and separated into plasma and cells by immediate centrifuging according to methods described in a preceding article (5). This procedure was followed because it has been demonstrated that there are changes in the phosphorus fractions if the serum is secured by slow coagulation. The method of analysis was similar to that outlined in a previous publication (1). The blood for calcium studies was similarly drawn but permitted to coagulate. The serum so obtained was analyzed by the methods outlined by Benjamin and Hess (6).

These analyses were repeated once a month or oftener beginning at the time the chickens were 1 month of age and continuing through the periods of growth, egg production, and subsequent molting. The results, which are graphically illustrated, present interesting and surprising data which demonstrate that the usual determination of only the inorganic calcium and inorganic phosphorus contents of the serum fails to present a true picture of the changes taking place in the blood of the fowl.

In Chart I are curves showing the fractions of phosphorus determined both in the cells and plasma throughout the experimental period. These percentages have been calculated in terms of parts of the total blood content and are represented as ordinates in the figure. The abscissa divisions show ages in terms of months. It will be noted that there is a gradual increase in the total phosphorus for the first 5 months and then a rapid rise at the time of production, this high level being held during the entire production period with some fluctuations and dropping quickly as production ceases and molting season approaches. This condition is true for

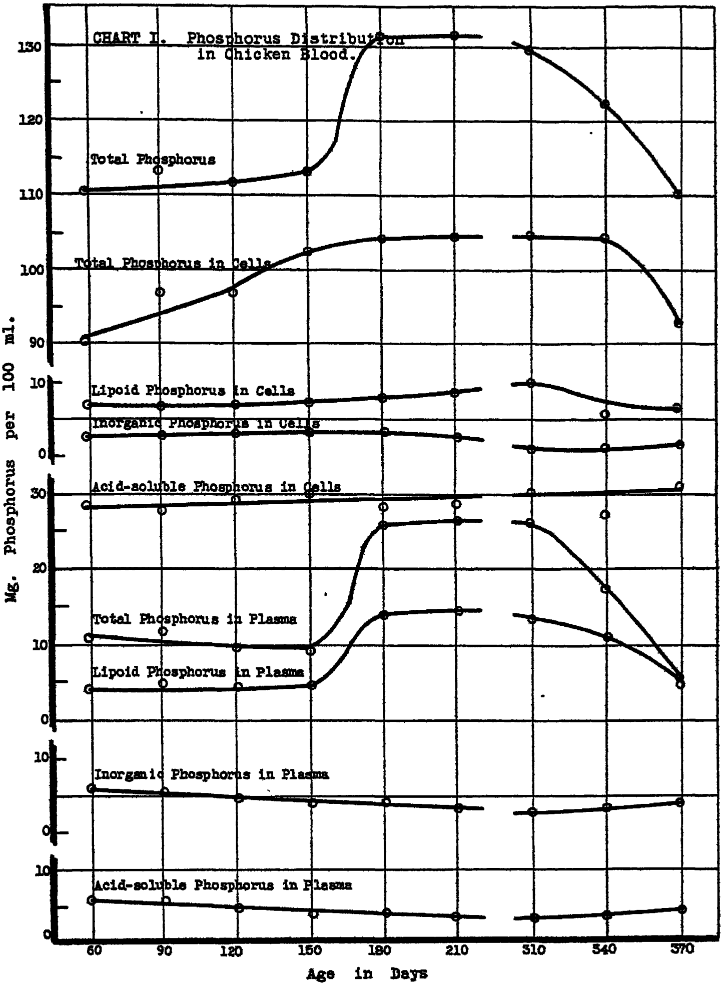


Chart I

total phosphorus of both cells and plasma. An examination of the other fractions reveals that this increase is due to the lipid fraction of both cells and plasma, while the inorganic and acid-soluble fractions remain rather fixed at all ages. We have previously called attention to the fact that this inorganic fraction, which is usually used in blood studies, does not give the true picture of phosphorus conditions. An examination of the above data reveals that the fraction which is responsible for changes must be the more complex lipid fraction. Had the inorganic fraction only been determined, as is the usual custom in making such studies, little noticeable change would have been observed throughout the entire life cycle, yet certain profound changes must have been taking place to cause the fluctuations as indicated by the lipoid phosphorus curves. These findings are somewhat contradictory to the theory often expressed that the inorganic fraction is responsible for calcification. Possibly the lipid could be considered as a storage form, but the quick changes occur so nearly at the same time as egg production that this theory is difficult to explain.

The blood for calcium studies was also obtained by heart puncture but was delivered into chilled flasks where it was permitted to coagulate normally. After 2 hours the coagulum was broken up and placed in tubes and the serum separated by centrifuging. The method of making calcium distribution determinations as outlined by Benjamin and Hess (6) was followed in detail.

The samples for analysis were obtained every 2 weeks from the time the chickens were 4 weeks old until they had passed through production and into the molting stage. It is apparent that the total calcium varies from 12 or less mg. per 100 ml. of serum for non-laying chickens to 24 mg. for laying hens. 2 weeks after production had started the total calcium was still rising slowly, as is illustrated in Curve 1 of Chart II. Curves 2 and 3 for protein-bound and total adsorbable calcium show the same general trend. However, when the total adsorbable calcium is divided into its components, the non-filtrable adsorbable calcium (Curve 5) and the filtrable adsorbable calcium (Curve 4), it is found that marked changes are taking place. The non-filtrable adsorbable complex is not present in significant quantities in the young hen, but makes its appearance about 8 weeks before egg production begins, at which time about 2 mg. per 100 ml. are present. At the time of production this value ascends rapidly to over 8 mg. The converse is true of the filtrable adsorbable calcium, the degree of variation being much less, which drops from 6 to less than 4 mg. per 100 ml.

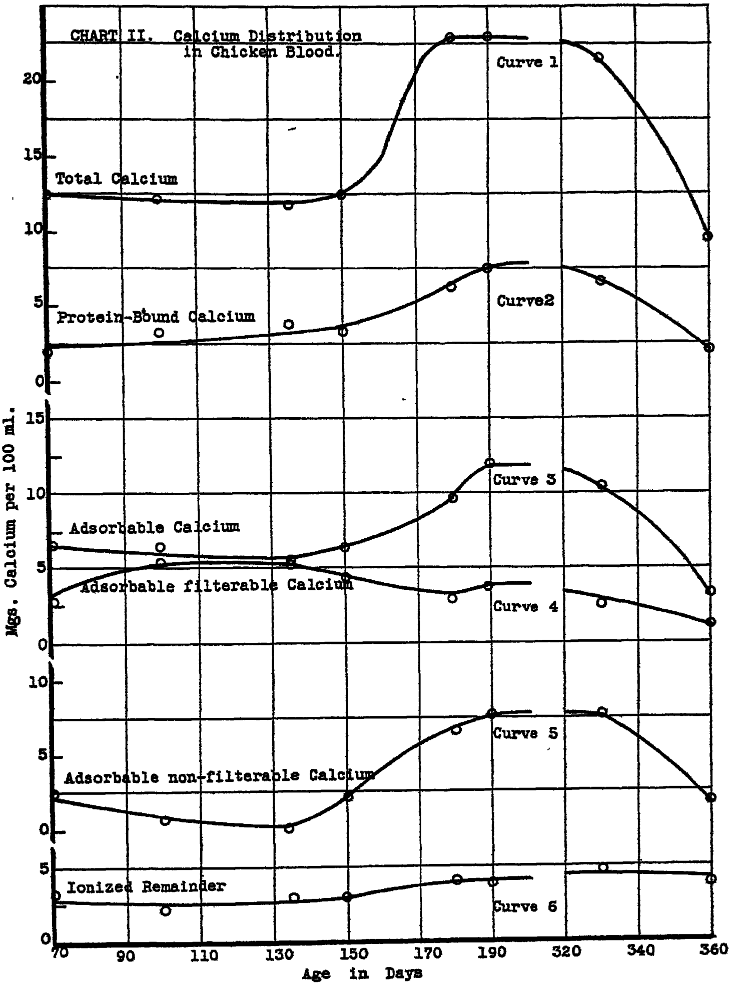


Chart II

(Curve 4). Curve 6 shows only a slightly upward trend for the ionic fraction with values varying from 2 to 4 mg. From these results it appears that the non-filtrable adsorbable complex form of calcium is the most closely associated with the process of egg-shell formation and it must be the fraction which is physiologically active and therefore responsible directly or indirectly for the larger portion of calcium used in egg production. If this fraction is the one that contributes to shell production there is evidence that it may also contribute to various forms of ossification. Again, as in the case of the determination of total inorganic phosphorus of the blood serum, the inorganic calcium as commonly determined does not fully picture all the changes taking place in the animal so that the determination of various fractions would better picture conditions accompanying malformation of bone.

The error is not as noticeable as with the phosphorus, however, as the inorganic calcium of serum measures practically all the calcium of the blood, since the cells are almost devôid of calcium and practically all forms are transformed into and accounted for by the usual method of determining inorganic calcium. This is not the case with phosphorus, as more phosphorus is present in cells than in serum and any hemolysis taking place causes the serum to be contaminated by the phosphorus of the cells. Furthermore, the common method of determining inorganic phosphorus accounts for only a small part of that present in plasma, as was more fully explained in previous articles.

Studies of Osteoporotic Chicks

An abnormal condition, often encountered in chicks kept in brooders, referred to by poultrymen as hock disease, porosis, or slipped tendons, and thought to be due to a deficient or unbalanced diet, has been extensively studied in this country. The symptoms in certain respects resemble those of rickets; consequently, many attempts have been made to diagnose this condition by blood and bone analyses as has been done in the study of rickets.

Many calcium and phosphorus ratios and distributions have been made in this laboratory during the past 3 years. It was found necessary to make the determinations reported in the first section of this paper due to unexplained changes in the blood analyses of the normal group. It having been established that there are normal changes taking place, caused by age, sex, and other factors, calcium and phosphorus distribution studies were made of groups of normal and osteoporotic chickens of definite sex and age, using methods previously mentioned. The averages of five or more sets of analyses are presented in Tables I and II.

Table I lists the calcium distribution of the blood of normal and osteoporotic chickens of the same age and origin. A comparison of the two sets of analyses is of interest because the total calcium contents of the serum of the two are apparently similar, although changes are noted in certain fractions. These changes are significant because the same trends are displayed by individual distributions within each group.

TABLE I

Calcium Distribution of Blood of Normal and Osteoporotic Chickens

Group of chickens	Ca per 100 ml. serum					
	Total	Protein-bound	Adsorbable	Adsorbable, filtrable	Adsorbable, non-filtrable	Ionized remainder
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Normal..............	15.1	2.9	8.5	2.0	6.5	3.7
Osteoporotic.........	14.0	2.9	7.6	4.4	3.2	3.5

TABLE II

Phosphorus Distribution of Blood of Normal and Osteoporotic Chickens

Values are given in mg. of P per 100 ml. of blood.

Group of chickens	Total P	Cell P				Plasma P			
		Total	Lipid	Inorganic	Acid-soluble	Total	Lipid	Inorganic	Acid-soluble
Normal...............	115.3	100.0	6.4	1.9	25.0	15.3	9.1	5.9	4.5
Osteoporotic..........	119.6	106.8	6.3	2.9	27.8	12.9	6.9	4.9	5.4

While the methods have not had sufficient application to make positive statements as to their value in predicting the occurrence of this malady, it is believed that information obtained by such analyses may be used in making changes in the rations in order to eradicate such conditions.

Table II presents the average phosphorus distributions of the cells and plasma of normal and abnormal 8 week-old chickens.

In general, statements made in regard to the calcium distributions hold for the phosphorus data, except that in the case of the phosphorus analyses the changes are observed in the totals and there are small changes in the inorganic fraction of the plasma, the only determination ordinarily made in most laboratories. It is believed that with the accumulation of such data, there will be found a close correlation between phosphorus and calcium partitions and abnormal bone developments.

SUMMARY

1. Analyses of complete calcium and phosphorus distribution in chicken blood have been made at regular intervals throughout the life cycle of the chicken.

2. It has been observed that there are definite changes not only in the totals but in certain partitions at various periods.

3. The variations in different fractions are not proportional, the adsorbable filtrable calcium decreasing at the time of egg production, while the other fractions greatly increase.

4. The totals for both calcium and phosphorus show pronounced increases at the time of egg production and return to former levels at molting time.

5. Comparison of the distributions of the blood of normal chickens and of those afflicted with osteoporosis reveals a significant tendency toward regular changes in certain fractions of both calcium and phosphorus, indicating the possibility of correlating such analyses and ossification.

BIBLIOGRAPHY

1. Heller, V. G., Hunter, K. R., and Thompson, R. B., *J. Biol. Chem.*, 97, 127 (1932).
2. Benjamin, H. R., and Hess, A. F., *J. Biol. Chem.*, 103, 629 (1933).
3. Correll, J. T., and Hughes, J. S., *J. Biol. Chem.*, 103, 511 (1933).
4. Paul, H., and Heller, V. G., *Proc. Oklahoma Acad. Sc.*, 14, 32 (1934).
5. Heller, V. G., and Paul, H., *J. Lab. and Clin. Med.*, 19, 777 (1934).
6. Benjamin, H. R., and Hess, A. F., *J. Biol. Chem.*, 100, 27 (1933).

RELATION OF DEPOT FAT TO EGG YOLK FAT IN LAYING HENS

By H. J. ALMQUIST, F. W. LORENZ, and BEN R. BURMESTER

(*From the Division of Poultry Husbandry, University of California College of Agriculture, Berkeley*)

(Received for publication, June 5, 1934)

It has been shown by Henderson and Wilcke (1933) that a fat-soluble dye (Sudan III), deposited in the fat reserves of laying hens, is not redeposited in the yolk fat under normal conditions. Lorenz, Almquist, and Hendry (1933) have shown that the Halphen test, which is characteristic of malvaceous fat, can be obtained from the yolk fat of eggs from hens which have eaten malvaceous plants, or products of these plants containing crude malvaceous fat (such as cottonseed meal or mallow (*Malva parviflora*) seed).

Cruickshank (1932) has reported that the iodine number of yolk fat and depot fat of hens may be raised by feeding a fat of a high iodine number. When fats of a low iodine number are fed, however, the depot fat of hens is decreased below normal, while that of the yolk fat is not correspondingly decreased below a normal value. In these respects it is evident that body fat and yolk fat do not follow the same metabolic paths. Fat deposits from different parts of a bird were found to be very similar in respect to the iodine number.

The following experiments were undertaken in an attempt to obtain further evidence on the possible utilization of depot fat in the formation of yolk fat, particularly from the standpoint of the fat-soluble substance which produces the Halphen test. The test for this substance is probably more sensitive than observations of dye color. In addition, it is believed that the Halphen test substance is an unsaturated fatty acid (Kuhn and Bengen, 1906). It can, therefore, be expected to follow the normal course of fat metabolism as well as, or better than, a foreign dye.

EXPERIMENTAL

Single comb white Leghorn pullets, hatched on the same date, were raised in batteries to 136 days of age, then transferred to an outside pen. Two birds were killed at this time and examined for the size of their ova, which were found to show no signs of growth preparatory to laying.

At the age of 152 days, seventeen pullets were leg-banded and placed on an all mash feed containing 1 per cent of crude cottonseed oil. This feed gave a strong Halphen test when tested according to the procedure in the "Official and tentative methods of analysis" (1931). Interfering green pigments also extracted from the feed by ethyl ether were removed without impairing the Halphen test by filtering the ether extract once or twice through bone-black.

At an age of 167 days, the seven most mature pullets were selected. Two of these were killed and the size of their ova determined. In one bird, an ovum 3 mm. in diameter was found; the remaining ova from both birds were very small. The ovaries gave negative Halphen tests, while the abdominal fat deposits gave distinct positive tests. The remaining five birds were placed in laying batteries and given a feed containing no cottonseed oil. This feed gave a negative Halphen test.

Halphen tests were made of the yolk fat of successive eggs laid by these birds until it was evident that the yolk fat was distinctly negative. Two pullets which had been on the cottonseed oil feed for a longer period of time were later added to this group. After the eggs of a given bird were found negative, the bird was killed and the ovary and abdominal fat removed for testing. Data from this group of birds are given in Table I.

Halphen tests of ovaries and yolks were made by allowing the wet material to stand overnight, or longer, with several volumes of ethyl ether (preferably alcohol-free). The clear extract was decanted off, and the ether evaporated on the water bath. To the residue were added 5 cc. of a 1 per cent solution of sulfur in carbon disulfide, and 5 cc. of isoamyl alcohol. The mixture was warmed gently at first and then heated for at least an hour in a saturated calcium chloride bath. When performed in this way, the test was found to develop a distinct pink tinge with amounts of cottonseed fat (the same fat as used in the feeds) as small as 1 part in 2500 parts of wet yolk, or approximately 1 part of cottonseed fat

TABLE I

Halphen Test Data on Depot Fat and Yolk Fat of Hens Fed Cottonseed Fat While Approaching Maturity

Hen No.	Age when killed	Weight of ovary	Halphen test of ovary	Weight of abdominal fat	Halphen test of abdominal fat	Age at which cottonseed fat mash was fed	Halphen tests on composite samples of eggs of birds at different ages			
	days	*gm.*		*gm.*		*days*	*days*	*days*	*days*	*days*
4465	136	0.24								
4524	136	0.30								
C-434	167	0.49	–		+	152–167				
C-442	167	0.63	–		+	152–167				
C-429	195		–		+	152–167	+ at 171,177,180	– at 183,185,187	– at 188,190,194	
C-437	195		–		+++	152–167	++ " 173,174,176	– " 178,181,183	– " 184,187,188	– at 189,193
C-444	209	46.3	–	46.7	+	152–167	– " 198,199,200			
C-441	230	33.0	–	51.4	–	152–167	– " 221,222,226			
C-445	230	34.0	–	23.2	+	152–167	– " 221,222,223	– at 224,228,230		
C-436	230	18.0	–	14.3	+++	152–209	– " 220,221,222	– " 226,228,230		
C-430	251	1.0	–	2.9	+	152–209	No eggs laid			

in 800 parts of ether-soluble yolk fat. Control mixtures having no cottonseed fat were negative to the test. For convenience and greater sensitivity, eggs were usually tested in groups of three.

At an age of 209 days the remaining birds in the outside pen, which were still being fed the cottonseed oil mixture, were operated upon, and all ova larger than $\frac{1}{4}$ inch in diameter removed. Eggs present in the oviduct were forced out. All of these birds had previously laid eggs giving positive Halphen tests, except for one border line case (Hen C-435). The ova removed by operation gave positive Halphen tests in every case. The birds were then given an all mash feed containing no cottonseed fat and having a negative Halphen test. Three of these birds laid eggs 12 days after the operation, while the last bird resumed production in 25 days. All eggs laid after the operation were negative. The birds were subsequently killed, and the ovaries and abdominal fat removed and tested as before.

Two birds of this second group were placed on a fat-free diet consisting of the usual all mash feed, which had been thoroughly extracted with ethyl ether. Vitamins A and D were restored to this feed by the use of ground medicinal cod liver oil concentrate tablets. Because of the dusty nature of this extracted feed, it was given to the birds at the rate of 100 gm. per bird per day, with sufficient water to make it similar to a wet mash. This experiment was made in an effort to cause a partial withdrawal of body fat for the formation of yolk fat. The eggs produced in this period (14 days) were negative to the Halphen test, as before. Data obtained in these experiments are summarized in Table II.

In certain cases, all other fat deposits were removed from the birds and found to give approximately the same response to the Halphen test as the abdominal fat. The results obtained with abdominal fat can, therefore, be regarded as representative of all depot fat.

Results

Hens C-429 and C-437 began laying 4 to 6 days after the feeding of the cottonseed oil mixture had been discontinued. The first eggs laid by these hens gave positive Halphen tests but tests on subsequent eggs soon became negative. Birds which started laying 21 to 54 days after removal of the cottonseed oil mixture

TABLE II

Halphen Test Data on Depot Fat and Yolk Fat of Hens Fed Cottonseed Fat from Age of 152 Days to 209 Days

Ova larger than $\frac{1}{4}$ inch in diameter were removed at the age of 209 days.

Hen No.	Age when killed	Weight of ovary	Halphen test of ovary	Weight of abdominal fat	Halphen test of abdominal fat	Halphen tests on composite samples of eggs of hens at different ages				
						Preoperative	Postoperative tests; all negative readings			
	days	*gm.*		*gm.*		*days*	*days*	*days*	*days*	*days*
C-431	230	24.6	−	25.5	+++	All + to 209	221,222,223	225,229,230		
C-439	230	28.7	−	17.1	++	" + " 209	224,225,226			
C-443	230	37.5	−	16.2	++	" + " 209	221,222,223	224,225,226		
C-432	251			25.6	+	" + " 209	226,229,233	234,237,242	243,244,245	
C-435	251			15.8	±	" ± " 209	233,234,237	243,247,248		
C-438	251			15.0	++	" + " 209	234,240,244	247,248		
C-433	251	10.0	−	18.5	±	" + " 209	229,233,234	238,*239,240	242,246,248	
C-440	251	15.3	−	29.6	++	" + " 209	221,229,233	234,*238,239	240,241,243	244,247,248

* Hens C-433 and C-440 were fed a fat-free mash from the age of 237 days to 251 days.

produced only yolks which gave negative tests. The yolks of eggs laid by Hens C-429 and C-437, which gave positive Halphen tests, were probably in the stage of rapid growth while these birds were still on the cottonseed oil feed. In all except one case (Hen C-441), positive tests were obtained from the abdominal fat when the bird was killed. The complete ovaries gave negative tests in every case.

All birds that were kept on the cottonseed oil feed until laying had started produced eggs which gave positive tests. When stripped by operative procedure of all maturing yolks larger than ¼ inch in diameter, and no longer fed the cottonseed oil, they consistently produced eggs with yolks giving negative tests. In six of these birds the abdominal fat gave strongly positive Halphen tests at the end of the experiment. All complete ovaries tested gave negative tests. One bird (Hen C-435) laying eggs which gave a doubtful test (indicated by ±) before operation, was also found to have a doubtful test in the abdominal fat when killed. There is, probably, a variation in the ability of different hens to deposit the Halphen test substance. The abdominal fat of Hen C-433, one of the birds kept on the fat-free diet, also gave a doubtful test. The abdominal fat of Hen C-440, the remaining bird on the fat-free diet, gave a strong positive test.

The failure of the Halphen test substance to appear in yolk fat of birds which were on a fat-free diet for 14 days is of particular significance. The increased metabolic difficulty imposed on these birds in the necessarily increased synthesis of yolk fat from carbohydrate and protein might have been expected to cause a partial employment of reserve body fat for the formation of yolk fat. However, such does not appear to be the case. Hen C-433 laid six eggs during this period, while Hen C-440 laid eight eggs; hence production was maintained at a satisfactory rate.

It is difficult to estimate the extent of dilution of the Halphen test substance when it is incorporated in depot fat, and the further dilution which may result if it is transferred from depot fat to yolk fat. The extreme sensitivity of the test indicates, however, that very little, if any, of the Halphen test substance in depot fat is involved in the formation of yolk fat. This agrees with the results of Henderson and Wilcke (1933) with Sudan III.

There seems no reason to believe that a special mechanism exists by which depot fat is freed from materials, such as Sudan III and the Halphen test substance, when used in forming yolks, since food fat is not similarly affected. These results strongly imply, therefore, that depot fat is not utilized in the formation of yolk fat.

The fact that three of the birds operated upon resumed production in 12 days is of particular significance in regard to the minimum time required to complete the formation of a yolk. Whether the longer time taken by the other birds was due to a slower rate of formation of yolk material, or to a slower recovery from the effects of the operation, cannot be decided.

SUMMARY

1. The fat-soluble substance responsible for the Halphen test is deposited in the depot fat and yolk fat of hens eating malvaceous plants, or products of these plants containing crude malvaceous fat.
2. When yolks present at the time of ingestion of malvaceous fat are removed by normal laying or by operative procedure, a positive Halphen test is not obtained in subsequently formed yolk fat, although the test may remain strongly positive in depot fat.
3. Depot fat is not utilized, to any important extent, in the formation of yolk fat.

BIBLIOGRAPHY

Cruickshank, E. M., *Harper Adams Utility Poultry J.*, **17**, 12 (1932).

Henderson, E. W., and Wilcke, H. S., *Poultry Sc.*, **12**, 266 (1933).

Kuhn, B., and Bengen, F., *Z. Untersuch. Nahrungs- u. Genussmittel*, **12**, 145 (1906).

Lorenz, F. W., Almquist, H. J., and Hendry, G. W., *Science*, **77**, 606 (1933).

Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 3rd edition, 230 (1931).

SPECTRAL ANALYSIS OF PURIFIED TUBERCULIN*

By MONA SPIEGEL-ADOLF and FLORENCE B. SEIBERT

(From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University School of Medicine, and the Henry Phipps Institute, University of Pennsylvania, Philadelphia)

(Received for publication, May 14, 1934)

The following experiments were undertaken in order to extend our knowledge of the chemistry of tuberculin. Studies of the absorption spectra of various tuberculin fractions in ultra-violet light were made, since the spectral test is known to be more sensitive in many cases than are analytical methods, and since such studies have proved to be of great value in investigations on the biologically active vitamins, insulin, etc. Because only reasonably pure substances can be studied by this method, the purified tuberculin products of protein nature prepared by Seibert and Munday (1, 2) were used.

Preparations Studied—Nine tuberculin preparations made from the human type tubercle bacillus, one each from bovine and avian bacilli, and one from each of the following non-pathogenic acid-fast bacilli, butyricum, smegma, and timothy, were studied. All of these products, except six in the case of the human type bacillus, were made by the method described by Seibert and Munday (1), which consisted, briefly, in washing and concentrating by ultrafiltration the culture filtrates from synthetic media, precipitating with trichloroacetic acid, washing extensively with the acid, and then removing the acid by means of large quantities of ether, which simultaneously dehydrated the precipitate to powder form. Three preparations (OTT, SOTT-4a, and SOTT-M-2) were made by this same method, except that a heated tuberculin, Koch's "old tuberculin," or an OT made on synthetic medium, was used

---

* Parts of this paper have been presented before the meeting of the Federation of American Societies for Experimental Biology at New York, March 29, 1934.

Aided by a grant from the National Tuberculosis Association.

as the source of the material. Two other preparations (M-9 and B-56) were made by precipitating the tuberculin protein by half saturation with ammonium sulfate (2), reprecipitating in this manner once in one case and four times in the other, and finally dialyzing. Another product (SOTTA) was made by combining the ammonium sulfate and trichloroacetic acid methods, precipitating SOTT-4a with ammonium sulfate, redissolving the precipitate in water, reprecipitating with trichloroacetic acid, washing with the acid until free of sulfate, and finally with ether to remove the acid. In addition to these tuberculin preparations, many other substances, which will be mentioned later, were studied during an attempt to identify the substance giving the special absorption band. In all, 360 spectrograms form the basis for the conclusions in this paper.

Apparatus and Methods Used

Solutions for spectral studies were made so that 1 cc. contained 10 mg. of a product and also 0.88 mg. of sodium hydroxide. Spectrograms were made of the preparations at concentrations ranging from 0.5 to 0.06 per cent. The hydrogen electrode was used for determining the pH of the solution.

The small Hilger quartz spectrograph which was used contained an internal wave-length scale that had been rechecked with the spectrum of a mercury-quartz lamp. The source of light was a clock-fed carbon arc lamp. Carbon B (National Carbon Company) containing iron provided the spectrum. Control tests were made with carbon C containing iron, nickel, aluminum, and silicon. A shutter permitting exposures of 0.04 second and less was used with the Wratten and Wainwright M plates. The quartz cell was 5 mm. in diameter.

The potency of preparations was determined by means of the Mantoux test and in some cases by the killing power in tuberculous guinea pigs. Some products were also tested on human beings.

Analyses for phosphorus content of different preparations were made by the Fiske and Subbarow method (3).

Results

The spectrographic analyses gave the following results. In the products made from the non-pathogenic bacilli, timothy, butyricum, and smegma, no special absorption band (Fig. 1) was present. All preparations made from human type tubercle bacilli, except two (M-9 and SOTTA), showed a selective absorption between 275 and 252 $m\mu$, with an average maximum at 267 to 265 $m\mu$, in addition to end-absorption (Fig. 2).

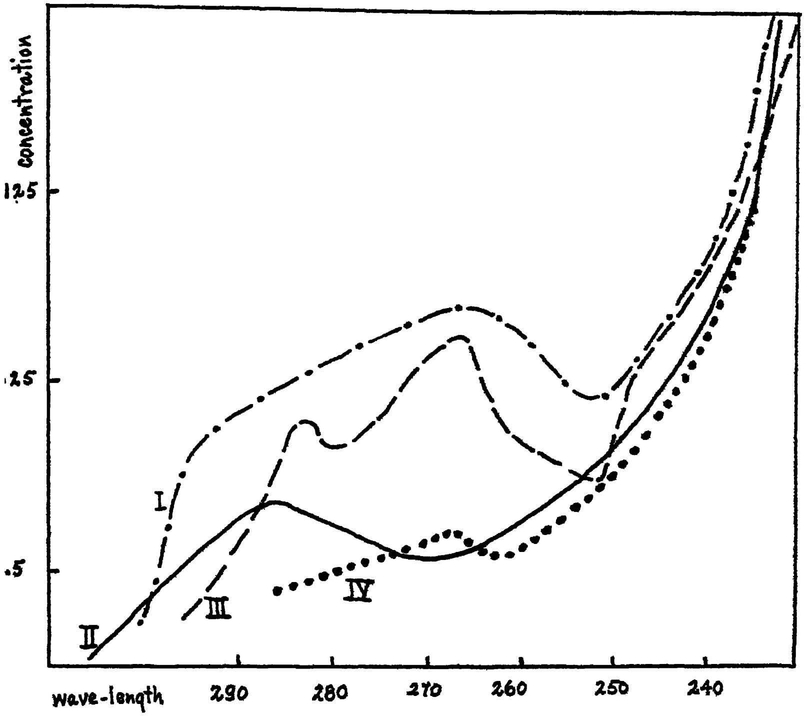


FIG. 1. Spectral absorption curves of various tuberculins. Curve I, TPT from bovine tubercle bacillus; Curve II, TPT from timothy bacillus; Curve III, OTT from "old tuberculin;" Curve IV, TPT from avian tubercle bacillus.

The preparations from the avian and bovine tubercle bacilli showed special absorption bands at the same wave-lengths as the human bacilli products, but in the case of the avian there was only a trace at the highest concentrations, whereas the bovine fraction compared closely with the human (Fig. 1).

In many cases the tuberculin skin potency of the preparations

TABLE I

Products from Human Tubercle Bacillus; Methods of Preparation, Properties, and Potencies

Preparation	Method of preparation*	Nature of product	Phosphorus	Potency of preparation in tuberculous guinea pigs	C_{OH} of solution tested
			per cent		
TPT-8	Pptd. by trichloroacetic acid	Mostly denatured protein	0.635	0.00001–0.00002 mg. gives skin reaction with necrosis; 1–2 mg. intraperitoneally = M.L.D. (24 hrs.)	3.59×10^{-4}
TPT-13	“ “	“ “		Equal to TPT-8 in skin potency	
TPT-20	Pptd. by trichloroacetic acid (no contact with phenol)	“ “		“ “	
OTT	Isolated from Koch's “old tuberculin” by trichloroacetic pptn.	Mostly proteoses	0.53	“ “	
SOTT-4a	Isolated from “old tuberculin” made on synthetic medium by trichloroacetic pptn.	“ “	0.57	Twice as potent as TPT-8 (as well as in clinical skin tests); 0.75 mg. lethal	
SOTT-M-2	Similar to SOTT-4a	“ “		Slightly less skin potency than SOTT-4a (also in clinical tests)	5.66×10^{-4}
B-56	Pptd. by $(NH_4)_2SO_4$ once (no contact with phenol)	“ undenatured protein; 25% polysaccharide	0.062	Potent in skin and lethal tests	

M-9	Pptd. by $(NH_4)_2SO_4$ ($\frac{1}{2}$ saturation) 4 times	Mostly undenatured protein; 2.8% polysaccharide	0.098	Equal (approximately) to TPT-8 (also in clinical skin tests); 5 mg. = M.L.D.	
SOTTA	SOTT repurified by $(NH_4)_2SO_4$ pptn. 4 times	Mostly denatured protein	0.30	Equal to SOTT-4 in skin potency; 0.75 mg. lethal	
Nucleic acid from human tubercle bacilli	Prepared by Coghill		8.11	0.1 mg. gave no skin reaction, 1 mg. only medium skin reaction; 5 mg. intraperitoneally not lethal	$C_{OH} = 2.09 \times 10^{-3}$ (0.066 N NaOH) $C_H = 2.13 \times 10^{-6}$ (0.021 N NaOH)

* All preparations except OTT were made on synthetic medium.

seemed to parallel the presence of the absorption band (Table I and Figs. 1 to 3). Note especially the trace of absorption by the avian fraction compared with its low degree of potency, the high degree of both potency and absorption of the bovine preparation, and the relative potency and absorption of SOTT-4a in comparison with TPT-8. SOTT-4a, which showed the special absorption band in half the concentration required by TPT-8, had twice the potency of the latter. Similarly TPT-8c which had been made (4)

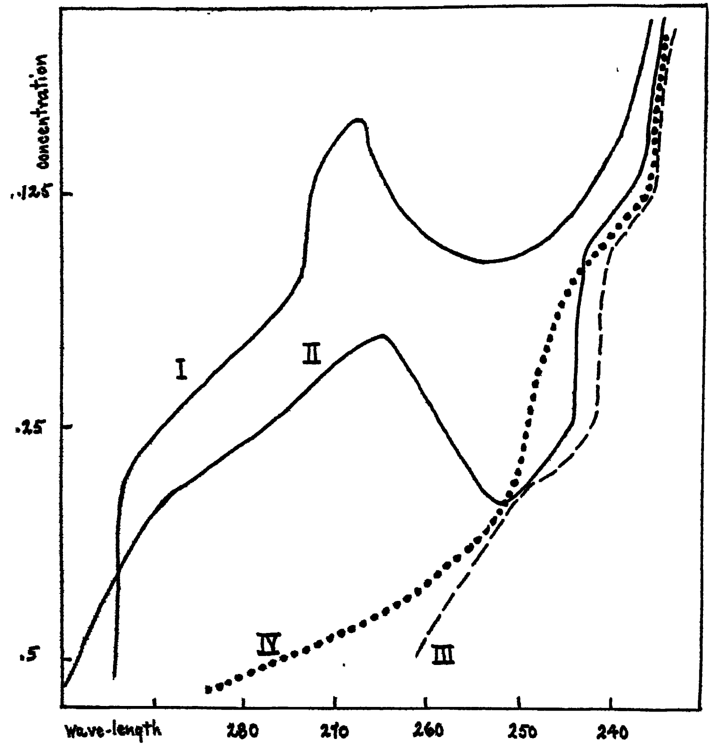


FIG. 2. Spectral absorption curves of various tuberculins from human type tubercle bacillus. Curve I, SOTT-4a, purified from synthetic "old tuberculin" by trichloroacetic acid method; Curve II, TPT-13, purified from unheated tuberculin by trichloroacetic acid method; Curve III, M-9, prepared by ammonium sulfate method; Curve IV, SOTTA, prepared from SOTT-4a by ammonium sulfate method.

by heating TPT-8 with alkali, lost half of its potency and showed a corresponding decrease in selective absorption.

Nevertheless, since two fractions from human type tubercle bacilli, M-9 and SOTTA, showed no special absorption bands and yet were no less potent than those showing the spectral absorption, it is evident that this property of absorbing light selectively does not belong to the active principle, but rather to an accompanying substance, which can be removed. In fact, in preparing SOTTA, the attempt was deliberately made to remove such a light-absorbing substance from SOTT-4a by using the method which had apparently succeeded in rendering M-9 free from spectral absorption. Therefore, combination of trichloroacetic acid and ammo-

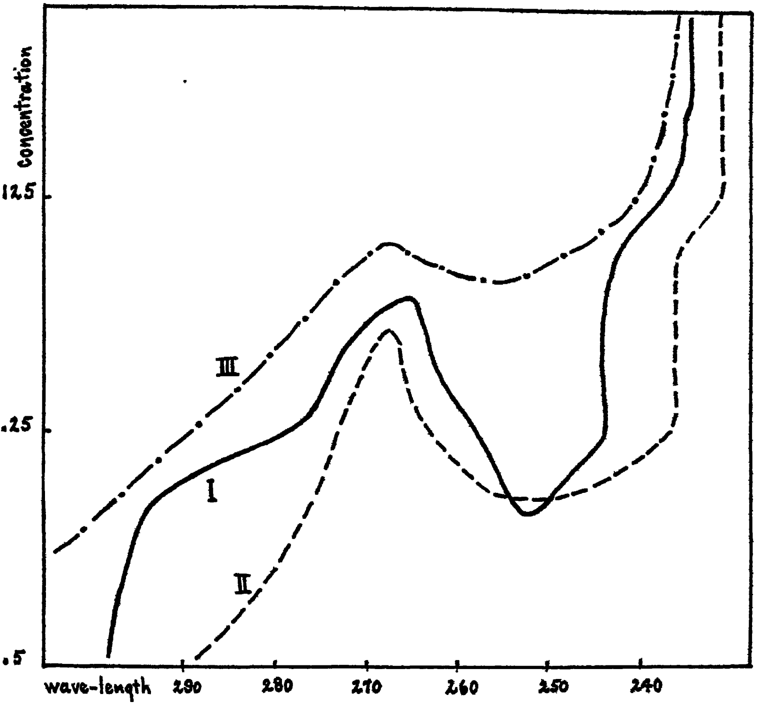


Fig. 3. Spectral absorption curves of products isolated from the human type tubercle bacillus. Curve I, TPT-8, tuberculin purified by trichloroacetic acid method; Curve II, nucleic acid; Curve III, SOTT-4a after irradiation for 10 hours.

nium sulfate precipitation effects an improvement in the purification methods of tuberculins.

Identification of Substance Giving Spectral Absorption

In an endeavor to determine the nature of this accompanying substance, the spectrographs of a great many compounds were studied under exactly the same conditions of concentration and alkali content. In this way the following substances were eliminated from consideration: phenol, which had been temporarily in contact with most of the preparations; glycerol, used in the culture medium; acetone, a possible intermediary metabolite; and the pigment phthiacol, isolated by Anderson and Newman (5) from tubercle bacilli.

Obviously it was important to determine whether the selective absorption of the tuberculins was due to some protein-like substances or protein derivatives, since most of the latter do show special absorption bands. Although the spectra of most of the substances studied are well known, we examined them again in the presence of the same amount of alkali as was used in the tuberculin solutions, since alkali is known to shift absorption spectra. Pseudoglobulin was found to absorb light selectively at a different wavelength (289–276 $m\mu$) and at a much higher concentration than did the tuberculins, and proteoses from Witte's peptone showed no selective absorption. The spectra of phenylalanine, which according to Tamura (6) is found in large quantity in the tubercle bacillus, of tryptophane, the concentration of which Kallós and Hoffmann (7) used as a measure of the potency of tuberculin, of lysine, found to be a cleavage product of tuberculosamine by Ruppel (8), of tyrosine, histidine, proline, and arginine, all proved to be entirely different from that found in the tuberculin fractions.

Ruppel (9) had shown that the tubercle bacillus contained a nucleic acid which Johnson and Brown (10) later demonstrated to correspond chemically to thymus nucleic acid. Since the nucleic acids and related compounds are known to give marked selective absorption, a study of such compounds was made under the conditions of the present experiments. The similarity of the absorption spectrum of thymus nucleic acid derived from fish sperm and that of some of the tuberculin preparations was remarkable. The nucleic acids isolated from the human, avian, and bovine tubercle

TABLE II

Characteristic Wave-Lengths of Spectrograms of Nucleic Acids and Their Constituent Pyrimidines

Thymine				Cytosine				5-Methylcytosine			
Concentration	**Absorption minimum**	**Range**	**Absorption maximum**	**Concentration**	**Absorption minimum**	**Range**	**Absorption maximum**	**Concentration**	**Absorption minimum**	**Range**	**Absorption maximum**
per cent				*per cent*				*per cent*			
0.027		312		0.025		286		0.05		294	
0.02	251	289	270	0.018	251	282	268	0.0375	254	289	270
		254				253				254	
		236				251				251	
0.0135	251	289	270	0.0125	251	273	268	0.025	254	285	270
		254				264				256	
		236				247				250	
0.01	251	273	269	0.009	251	273	268	0.01875	254	273	270
		265				264				265	
		231				236				240	
0.0067	251	273	269	0.0062		232		0.0125		236	
		265									
		231									
0.005	251	273	269	0.0045		231		0.0093		234	
		265									
		228									
Uracil				**Human tubercle bacillus nucleic acid**				**Timothy bacillus nucleic acid**			
0.034		298		0.06		287		0.06		285	
0.025	239	297	270	0.04	252	276	265	0.04	239	276	270
		243				254				243	
		236				251				236	
0.017	239	276	265	0.03	251	273	267	0.027	(251)	(273)	(267)
		254				264				(264)	
		235				236				232	
0.0125	(251)	232	268	0.02		236		0.0235		231	
0.0085		228		0.015		231		0.0135		228	
0.0062		227		0.01		231		0.01		228	

Parentheses indicate faint absorption.

bacilli and from the timothy bacillus, as well as the pyrimidines, thymine, uracil, and 5-methylcytosine, all of which were generously given to us by Dr. R. Coghill of Yale University, showed selective absorption. (Table II.) There was a marked difference between the absorption bands due to the nucleic acid isolated from the human type tubercle bacillus and that from the timothy bacillus, a difference which was analogous to that found between thymine and uracil. This is in agreement with Coghill's (11) finding that the nucleic acid obtained from the human type tubercle bacillus contains thymine, whereas that from the timothy bacillus contains uracil. Dhéré (12) had already pointed out that the absorption of a nucleic acid conforms to the absorption of its pyrimidines.

The correspondence between the spectra of human tuberculin (TPT-8) and that of the nucleic acid isolated from the human type tubercle bacillus, even when the alkali content was varied, was very striking, as seen in Fig. 3, indicating that the substance responsible for the selective absorption in the tuberculin is nucleic acid or at least a pyrimidine derivative of the latter. Moreover, the concentrations of the two solutions at which the bands appear, suggest that the nucleic acid is present to the extent of 10 to 12 per cent in the tuberculin. This is significant in view of the findings of Osborne and Harris (13), *viz.* that nucleic acids (from wheat and salmon) are able to combine with up to 6 molecules of protein.

TABLE III

Phosphorus Content of Several Tuberculins

Preparation....................	Bovine TPT	Avian TPT	Butyricum TPT	Timothy SOTT	TPT-8c
P content..............	0.51	0.025	0.02	0.108	0.56

Phosphorus, which is an easily detectable constituent of nucleic acids, was therefore determined in the case of most of the preparations. These analyses showed some correspondence between content of phosphorus and spectral absorption, except in preparation B-56 (see Tables I and III). On the other hand, the potency and content of phosphorus were not necessarily parallel. See especially fractions M-9 and SOTTA in relation to SOTT-4a (Table I).

The phosphorus data, therefore, like the evidence from the spectral absorption studies, support the idea that the substance in some tuberculin preparations responsible for absorbing ultra-violet light selectively is nucleic acid or a derivative, and that it is possible, although difficult, to remove it without destroying the biological activity of the tuberculin. These findings supplement those of Ruppel (9) and Kallós and Hoffmann (7) as to the presence of nucleic acid in tuberculin fractions, but do not support the statements of these authors and of von Behring (14), Kitajima (15), Römer (16), and others that the potency is dependent upon its presence. The results are in accord with findings of Long (17) and of Heidelberger and Menzel (18).

TABLE IV

Correlation of Tuberculin Potency with Degree of Irradiation

Duration of irradiation at 15 cm.	Dose for skin test	Degree of skin reactions on both sides of same tuberculous guinea pigs		Intraperitoneal dose	Result of lethal test
		Irradiated sample	Non-irradiated control		
	mg.			*mg.*	
TPT-8, 15 min.	0.0002	5+, 5++	5+, 5+	2	Died in 24 hours
	0.0001	5+, 5+	5-6+, 5-6+		
" 2 hrs.	0.0002	Trace, 2-3+	4+, 3+		
	0.0001	2+, 4-5+	4+, 5+		
" 4 "	0.0002	5–, 4+	5+, 5+	2	" "
	0.0001	5+, 5+	5+, 5+		
" 6 "	0.002	1-2+, 1+, 1+, –, –	5-6+, 5-6+, 5-6+, 5-6+, 5-6+	3	No reaction; lived
				4	" "
	0.0002	1+, 3+	4+, 4+		
	0.0001	2+, –, –	4+, 4-5+, 3-4+		
SOTT-4a, 10 hrs.	0.01	–, –, –, –	3-4+, 4+, 5+, 5-6+		
	0.1	–	5+		

The isolated purified nucleic acid from the human type tubercle bacillus is practically non-potent; 2000 times as much of this substance was required to produce a skin reaction in tuberculous guinea pigs as of the purified tuberculin fraction. This agrees fairly well with the results of Ruppel and Joseph (19).

Effect of Irradiation on Selective Absorption and Potency

The effect of irradiation with ultra-violet light upon purified tuberculin preparations was determined, since Fischer and Hausmann (20) had shown that the biological potency of OT, although resistant to heat, was destroyed in this manner. It was found that when the preparations TPT-8 in 0.1 and 1 per cent concentrations and SOTT-4a in 1 per cent concentration were irradiated in a quartz-covered dish at a distance of 15 cm. from the quartz burner for 6 to 10 hours, the special absorption band was destroyed and the toxicity, as measured by the intradermic test and killing power in tuberculous guïnea pigs (see Table IV and Fig. 3), was lost. The total absorption was increased, so that in intermediate stages of irradiation the absorption was similar to nonirradiated solutions of higher concentrations. Such augmentation of absorption in proteins after irradiation was described by Spiegel-Adolf and Krumpel (21), but no changes in absorption bands could be detected if protein cleavage was avoided. The loss of the special absorption band in the tuberculin fractions is consistent with the observations of Heyroth and Loofbourow (22), that irradiated uracil and related compounds lose their selective absorption.

We wish to acknowledge our indebtedness to Dr. J. Fanz (Philadelphia) and Professor O. Krumpel (Vienna) for the loan of some apparatus, and for checking the spectrographic results.

SUMMARY

Ultraspectrographic investigations made on nine samples of tuberculin of human type tubercle bacilli have shown that all the preparations made by trichloroacetic acid precipitation contained a substance showing selective absorption with a maximum at 265 to 267 $m\mu$. The substance giving this selective absorption could be identified by this spectral method with thymus nucleic acid. One tuberculin preparation made by repeated ammonium sulfate fractionation and another in which the same procedure was used on a trichloroacetic acid precipitate did not show any trace of nucleic acid spectrographically. The phosphorus content of these two preparations was markedly lower than that of the trichloroacetic acid precipitate. The biological potency was not impaired by the removal of the substance giving selective absorption. According to these results it is concluded that thymus nucleic acid is not identical with the potent principle of tuberculin, as was once

maintained by certain investigators. On the other hand, there seems to be some kind of an association between the nucleic acid and the potent principle. Tuberculins made from different types of pathogenic acid-fast bacilli showed a close correspondence between the biological potencies and their content of nucleic acid. No trace of nucleic acid could be detected by ultraspectrographic methods in preparations made from certain non-pathogenic acid-fast bacilli, *viz.* timothy, butyricum, and smegma bacilli. Procedures diminishing or destroying the potency of tuberculins, such as heating with alkali or irradiation with ultra-violet light, had a corresponding effect on the selective absorption.

BIBLIOGRAPHY

1. Seibert, F. B., and Munday, B., *Am. Rev. Tuberc.*, **25**, 724 (1932).
2. Seibert, F. B., and Munday, B., *Am. Rev. Tuberc.*, **23**, 23 (1931).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
4. Seibert, F. B., *Nat. Tuberc. Assn., Tr. 29th Ann. Meeting*, 165 (1933).
5. Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, **103**, 197 (1933).
6. Tamura, S., *Z. physiol. Chem.*, **87**, 85 (1913).
7. Kallós, P., and Hoffmann, G., *Biochem. Z.*, **266**, 132 (1933).
8. Ruppel, W. G., *Z. med. Chem.*, **2**, 18, 28 (1924).
9. Ruppel, W. G., *Z. physiol. Chem.*, **26**, 218 (1899).
10. Johnson, T. B., and Brown, E. B., *J. Biol. Chem.*, **54**, 731 (1922).
11. Coghill, R. D., *J. Biol. Chem.*, **90**, 57 (1931).
12. Dhéré, C., Thèse de Fribourg (1909).
13. Osborne, T. B., and Harris, I., *Z. physiol. Chem.*, **36**, 122 (1902).
14. von Behring, E., *Ber. klin. Woch.*, **36**, 577 (1899); *Behringwerk Mitt.*, **2**, 81 (1907).
15. Kitajima, T., *Centr. Bakt., 1 Abt., Ref.*, **33**, 727 (1903).
16. Römer, P. H., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, **1**, 1072 (1907–11).
17. Long, E. R., *Am. Rev. Tuberc.*, **4**, 842 (1921).
18. Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, **104**, 655 (1934).
19. Ruppel, W. G., and Joseph, K., *Z. Immunitätsforsh. u. exp. Therap., Orig.*, **21**, 277 (1914).
20. Fischer, A., and Hausmann, W., *Z. Tuberk.*, **58**, 328 (1930).
21. Speigel-Adolf, M., and Krumpel, O., *Biochem. Z.*, **208**, 45 (1929).
22. Heyroth, F. F., and Loofbourow, J. R., *J. Am. Chem. Soc.*, **53**, 3441 (1931).

A CONVENIENT SYNTHESIS OF *dl*-LYSINE

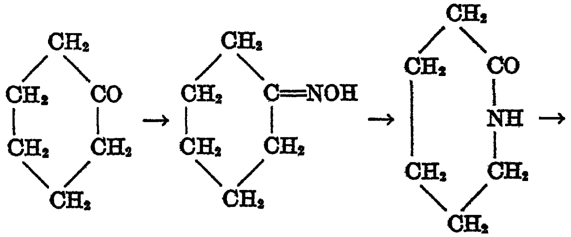
BY JOHN C. ECK AND CARL S. MARVEL

(*From the Chemical Laboratory of the University of Illinois, Urbana*)

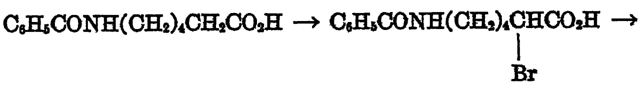
(Received for publication, May 31, 1934)

Lysine, *d*-α,ε-diaminocaproic acid, is one of the amino acids which is essential for the animal diet. It can be isolated as the picrate from protein hydrolysates but even the best procedure is laborious and the amino acid is expensive. Four complete syntheses of the *dl*-amino acid have been reported in the literature. Fischer and Weigert (1) obtained *dl*-lysine by a complex synthesis starting with γ-chlorobutyronitrile but the yields were not good. Sörensen (2) used cyanopropylphthalimidomalonic ester as a starting material in his synthesis of lysine but the procedure was too complex for practical production of the amino acid. Von Braun (3) synthesized *dl*-lysine by a 7-step method from piperidine. His procedure is not satisfactory because of the difficulty in cleaving benzoylpiperidine to obtain a good yield of benzoylaminoamyl chloride. Sugasawa (4) has more recently described a synthesis of *dl*-lysine from acrolein, but ten reactions are involved and, at best, the yields of final product are low, based on the starting material.

A very convenient synthesis of *dl*-lysine dihydrochloride from cyclohexanone has now been devised. The steps involved in this synthesis are as follows:

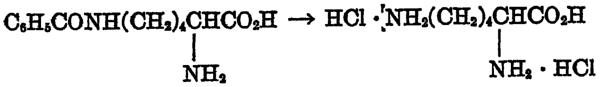


I II III



$$C_6H_5CONH(CH_2)_4CH_2CO_2H \rightarrow C_6H_5CONH(CH_2)_4\underset{\displaystyle Br}{\underset{|}{C}}HCO_2H \rightarrow$$

IV V



$$C_6H_5CONH(CH_2)_4\underset{\underset{\displaystyle NH_2}{|}}{C}HCO_2H \rightarrow HCl\cdot NH_2(CH_2)_4\underset{\underset{\displaystyle NH_2\cdot HCl}{|}}{C}HCO_2H$$

VI VII

None of these reactions is distinctly new but the details of the various steps have been worked out so that it is now possible to obtain approximately 500 gm. of *dl*-lysine dihydrochloride from 1000 gm. of technical cyclohexanone and no expensive reagents are needed in any stage of the synthesis.

Cyclohexanone (I) was converted to the oxime (II) by the general procedure which Semon (5) has developed for acetoxime. The oxime may be prepared by the standard procedure (6) but, although the yields are better, the cost is increased because of the expense of hydroxylamine hydrochloride. The oxime was rearranged to 2-ketohexamethyleneimine (III) by Ruzicka's (7) modification of Wallach's (8) method, and without isolation this lactone was hydrolyzed and benzoylated to give ε-benzoylaminocaproic acid (IV). The conversion of ε-benzoylaminocaproic acid to *dl*-lysine dihydrochloride was accomplished by the excellent method of von Braun (3).

EXPERIMENTAL

Cyclohexanoneoxime—In a 5 liter 2-necked, round bottomed flask fitted with an efficient mechanical stirrer and an 8 mm. glass inlet tube reaching to within 2 inches of the bottom of the flask, were placed a mixture of 182 gm. (2.5 moles) of technical sodium nitrite (95 per cent) and 2 kilos of cracked ice. The flask was placed in a cooling bath containing an ice-salt mixture. A cold (−8°) solution of sodium bisulfite, prepared by saturating a solution of 143 gm. (1.35 moles) of anhydrous sodium carbonate in 600 cc. of water with sulfur dioxide was added to the reaction flask. While the temperature was held below 0°, a moderate stream of sulfur dioxide was passed into this solution until it was acid to Congo paper and then enough longer just to remove the dark color which appears just before the solution becomes acid.

To this solution were added 196 gm. (2 moles) of technical cyclohexanone and 500 cc. of 95 per cent ethyl alcohol. The reaction mixture was heated on a steam bath to about 75° and then packed in mineral wool so that it cooled slowly. Rapid stirring was maintained during about 48 hours. The solution was then cooled to room temperature and exactly neutralized to litmus with a 50 per cent solution of sodium hydroxide. (This required about 165 gm. of sodium hydroxide.) The solution was stirred and cooled during the neutralization. The oily layer was separated and the aqueous solution was extracted with two 200 cc. portions of ether. The oily portion and the ether extracts were combined and distilled from a modified Claissen flask under reduced pressure. The product boiling at 95–100° under 5 mm. pressure was chilled and washed with a small volume of low boiling petroleum ether to remove oily material which apparently was produced by a condensation of cyclohexanone with itself. The yield of purified cyclohexanoneoxime, m.p. 86–88°, was 136 gm. (60 per cent of the theoretical amount). The quality of the oxime was very important, as a poor grade produced an inferior grade of ϵ-benzoylaminocaproic acid and the remaining steps in the synthesis were unsatisfactory.

ϵ-Benzoylaminocaproic Acid—The rearrangement of the oxime had to be carried out in 10 gm. portions, as the reaction was violent. 10 gm. of the oxime were placed in a 300 cc. Erlenmeyer flask and 20 cc. of sulfuric acid (sp.gr. 1.783, prepared by mixing 5 volumes of concentrated sulfuric acid and 1 volume of water) were added. The flask was heated over a low flame until bubbles first appeared. The flask was set aside until the violent reaction, which lasted a few seconds, had subsided. The acid solutions from ten such reactions (100 gm. of cyclohexanoneoxime) were combined in a 5 liter flask and diluted with 2.5 liters of water. This solution was gently boiled for about 1½ hours with about 5 gm. of decolorizing carbon (norit) in order to hydrolyze the lactam and to decolorize the solution. After this time the solution was filtered and exactly neutralized to litmus with 50 per cent sodium hydroxide solution. This usually required about 255 gm. of sodium hydroxide. The pale brown color which appeared on neutralization was then removed by boiling the solution for about ½ hour with about 5 gm. of decolorizing carbon.

The filtrate was placed in a 5 liter round bottomed flask fitted with a mechanical stirrer and cooled in an ice bath. The solution was made alkaline by the addition of a solution of 55 gm. of sodium hydroxide and then 94 gm. of benzoyl chloride were added dropwise from a separatory funnel. During the benzoylation the reaction mixture was well stirred and the temperature was kept at about 10°. The mixture was stirred for about an hour after the last of the benzoyl chloride had been added and then filtered and placed in a 4 liter beaker. The cold filtrate was slowly acidified to Congo red by the addition of dilute hydrochloric acid (about 10 per cent). The ε-benzoylaminocaproic acid was collected on a suction filter and when quite dry was washed with two 100 cc. portions of low-boiling petroleum ether to remove any benzoic acid. The product was then dried in a vacuum desiccator over sulfuric acid. The yield of product melting at 77–80° was 150 gm. (72 per cent of the theoretical amount based on the oxime).

α-Bromo-ε-Benzoylaminocaproic Acid (*3*)—An intimate mixture of 150 gm. of dry ε-benzoylaminocaproic acid and 26.4 gm. of dry red phosphorus was placed in a 1 liter 3-necked flask which was provided with a separatory funnel, an air condenser which was connected through a calcium chloride tube to a water trap, and a mechanical stirrer. The reaction flask was surrounded by an ice-salt mixture and the stirrer was started. The stirrer had to be very powerful because the mixture became very viscous during the reaction. To the cold mixture 408 gm. of dry bromine were added dropwise from the separatory funnel. When all of the bromine had been added the cooling bath was removed and the reaction mixture was slowly warmed and finally heated in a boiling water bath until the bromine vapors practically disappeared. The hot mixture was poured onto cracked ice in a 1 liter beaker and any excess bromine was reduced by passing in a slow stream of sulfur dioxide. The lumps of material were broken up to aid in the purification. The material which remained in the reaction flask was also treated with water and sulfur dioxide. The solid product was collected on a suction filter, washed with three 50 cc. portions of water, and air-dried. The yield of bromo acid melting at 163–166° was 180 gm. (90 per cent of the theoretical amount).

ε-Benzoyllysine—A solution of 180 gm. of the bromo acid in 2 liters of concentrated aqueous ammonia (sp.gr. 0.9) was allowed to stand for 2 days in a 5 liter flask. The solution was filtered to remove any suspended matter and the filtrate was evaporated to about 1 liter from a steam bath under reduced pressure. On cooling, the first crop of crystals of ε-benzoyllysine separated and this was collected on a suction filter and washed with about 100 cc. of alcohol and 100 cc. of ether. The aqueous filtrate from this portion was evaporated to dryness under reduced pressure and the residue was washed with two 100 cc. portions of water to remove the ammonium bromide. The benzoyllysine was then washed with about 50 cc. of alcohol and 50 cc. of ether.

The total yield of ε-benzoyllysine melting at 268–270° was 100 gm. (70 per cent of the theoretical amount).

dl-Lysine Dihydrochloride—A solution of 100 gm. of ε-benzoyllysine in a mixture of 600 cc. of concentrated hydrochloric acid (sp.gr. 1.19) and 400 cc. of water was boiled under a reflux condenser for 10 hours. The mixture was cooled and the benzoic acid was removed by filtration. The filtrate was evaporated under reduced pressure from a water bath until a thick syrup remained. This syrup was transferred to a 1.5 liter beaker by means of about 4 volumes of absolute alcohol. The alcoholic solution was cooled and 500 cc. of ether were added slowly while the mixture was stirred. The lysine dihydrochloride was collected on a suction filter. The yield was 75 gm. (85 per cent of the theoretical amount) of a product which melted at 188–190°. This product was analytically pure lysine dihydrochloride.

Analysis—$C_6H_{16}O_2N_2Cl_2$. Calculated. Cl, 32.42
Found. " 32.61, 32.62

SUMMARY

The details for the synthesis of *dl*-lysine dihydrochloride from cyclohexanone have been described. The over-all yield in this process is between 22 and 23 per cent of the theoretical amount, that is, 1000 gm. of cyclohexanone will yield about 500 gm. of *dl*-lysine dihydrochloride.

BIBLIOGRAPHY

1. Fischer, E., and Weigert, F., *Ber. chem. Ges.*, **35**, 3772 (1902).
2. Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **6**, 1 (1903).
3. von Braun, J., *Ber. chem. Ges.*, **42**, 839 (1909).
4. Sugasawa, S., *J. Pharm. Soc. Japan*, **550**, 1044 (1927).
5. Semon, W. L., *J. Am. Chem. Soc.*, **45**, 188 (1923).
6. Bousquet, E. W., in Marvel, C. S., *et al.*, Organic syntheses, New York, **11**, 54 (1931).
7. Ruzicka, L., *Helv. chim. acta*, **4**, 472 (1921).
8. Wallach, O., *Ann. Chem.*, **312**, 179 (1900).

THE ERGOT ALKALOIDS

III. ON LYSERGIC ACID

By WALTER A. JACOBS AND LYMAN C. CRAIG

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 13, 1934)

The production of an acid $C_{16}H_{16}O_2N_2$, lysergic acid, by the alkaline cleavage of ergotinine has been described in a previous communication.[1] At the time we had commented on the failure to recover from the reaction mixture the base, ergine, which had been previously isolated by Smith and Timmis[2] by the action of methyl alcoholic alkali on ergotinine. We stated that this failure

> "might have been due to the fact that although it [ergine] could have been formed during the reaction in aqueous alkali it might have been further degraded to lysergic acid. In order to determine this point we have replaced ergotinine in the above procedure by ergine. Although a crystalline acid was obtained in small yield it appeared on analysis to be definitely different from lysergic acid. With the amount available it was possible to give it but preliminary study."

At the time we had also noted that ammonia was formed by the action of alkali on ergine. Since then we have found that the substance which we had obtained was a sparingly soluble sulfate of lysergic acid and the analytical figures secured with this material naturally caused confusion in the original attempt to demonstrate the formation of lysergic acid from ergine.

In the meantime, Smith has written us that since their original communication on ergine, Timmis and he[3] have found that ergine does not possess the formula $C_{17}H_{21}ON_3$ originally derived by them, but $C_{16}H_{17}ON_3$ and that it is the amide of an acid $C_{16}H_{16}O_2N_2$ doubtless identical with lysergic acid. Our recent results confirm this view and have demonstrated definitely that the acids obtained by the action of aqueous alkali on ergotinine and ergine are identical.

---

[1] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **104**, 547 (1934).

[2] Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 763 (1932).

[3] Smith, S., and Timmis, G. M., *Nature*, **133**, 579 (1934).

In an earlier communication[4] we have reported the formation of *p*-nitrobenzoic acid and an acid $C_{14}H_9O_8N$ during the oxidation of ergotinine with nitric acid. Since the latter contains still the N-methyl group of ergotinine, it was important to determine whether lysergic acid would yield the same acid. On treatment with nitric acid under conditions similar to those formerly employed, no *p*-nitrobenzoic acid could be obtained and none of the above acid could be detected. However, a new acid has been isolated in exceedingly poor yield which on analysis gave figures from which a formula $C_{13}H_8O_8N_2$ has been derived. It still contains the N-methyl group, and the formation of a deep red color in alkaline solution, which again changed back to a pale yellow on acidifying, suggests the presence of a nitro group. The costliness of the material has prevented its further investigation.

It would appear that this acid may be related to that first obtained from ergotinine by the replacement of a carboxyl by a nitro group, and that in the formation of lysergic acid the bridge joining its precursor to the rest of the molecule in ergotinine may be ruptured in a different way under the influence of alkali from that which occurs on direct oxidation with nitric acid and which leads to a carboxyl group. At this point in lysergic acid a nitro group, instead of a carboxyl group, could be introduced. It is not excluded that the isobutyryl formic acid also produced by alkali may be involved in these considerations. We shall attempt to check such a possibility in a further study.

Since lysergic acid is a nitrogen heterocyclic derivative, it was of interest to study its behavior towards sodium in amyl alcohol. This procedure resulted in the formation of a new crystalline substance which still possesses acid and basic properties and is more stable than lysergic acid itself. From the analysis it appears to be a *dihydrolysergic acid*, $C_{16}H_{18}O_2N_2$. This formula was confirmed by the formation of a *methyl ester* by the use of methyl alcoholic hydrogen chloride.

At this point it should be mentioned that the usual tests for a primary or secondary amine grouping made on the ester of lysergic acid have failed. Attempts to acetylate the ester or combine it with phenyl isothiocyanate were negative. When boiled in toluene solution with metallic sodium, the compound did not appear to form a sodium derivative. It seemed possible that in the formation of the dihydro derivative reduction of one of the double bonds in the ester might produce a secondary amine, but this did not prove to be the case. The ester of dihydrolysergic acid does not react with phenyl isothiocyanate or acetyl chloride, and boiling with acetic anhydride gave only unchanged ester. However, the methyl ester of lysergic acid gave methane easily in the Zerewitinoff test for active hydrogen.

---

[4] Jacobs, W. A., *J. Biol. Chem.*, **97**, 739 (1932).

Finally, since lysergic acid contains one carboxyl group, it was of interest to investigate its behavior on dry distillation. When heated in a sublimation apparatus at 0.2 mm., a volatile base began to sublime when the bath reached 200° and attained a maximum at 250°. This base formed yellow leaflets and proved to be very unstable, so that during the attempts to recrystallize it decomposition interfered. Although the analytical figures were not conclusive, the formation of the base by loss of CO_2 was suggested. Since the amount of material available is so limited, a further study of this substance has been deferred.

Continued investigation of the degradation of lysergic acid is in progress.

Thus far it is apparent that the ergotinine molecule is made up of distinct portions, and it is probable that in the interconnection of some of them amide linkages play a rôle. These portions are represented by lysergic acid, isobutyryl formic acid, and perhaps by a benzyl or related grouping and a still undetermined nitrogen heterocyclic group. The fact that these alkaloids on decomposition with alkali liberate apparently only 1 mole of ammonia (Soltys[5]) raises the question whether the inferred labile amide linkage occurs in the alkaloid itself as a $CO \cdot NH_2$ group or a $—CO \cdot N \cdot H \cdot CO—$ group. Two different amides have been obtained by different procedures from ergotinine, namely ergine and isobutyryl formamide. It is obvious that the $CO \cdot NH_2$ groups in both of these cannot occur as $CO \cdot NH_2$ groups together in the alkaloid molecule.

---

[5] Soltys, A., *Ber. chem. Ges.*, **65**, 553 (1932).

EXPERIMENTAL

Ergine—Ergine was prepared according to Smith and Timmis[2] and recrystallized from methyl alcohol.

The analysis was made on the air-dried substance.

$C_{16}H_{17}ON_3 \cdot CH_3OH$.	Calculated.	C 68.18,	H 7.07,	N 14.04
	Found.	" 68.40,	" 6.99	
	"	" 68.49,	" 6.88	
	"			N 14.12
	"			" 14.20

Hydrolysis of Ergine—100 mg. of ergine were treated with 3 cc. of N sodium hydroxide and the mixture was heated on the steam bath in a nitrogen atmosphere for 80 minutes. The nitrogen gas from the reaction was passed through 10 cc. of 0.1 N sulfuric acid. On back titration 3.64 mg. of liberated ammonia were determined. The ammonia formed was identified by acylation with *p*-nitrobenzoyl chloride. The *p*-nitrobenzamide obtained melted at 198°.

The aqueous alkaline hydrolysate contained 20 mg. of unchanged ergine as a suspension which was filtered off through a sintered glass filter. The filtrate was neutralized to Congo red with sulfuric acid. After chilling and filtering, 80 mg. of dark colored crystalline material were obtained. This was suspended in 2 cc. of methyl alcohol and treated with 2 drops of ammonium hydroxide. The filtrate on evaporation to dryness under diminished pressure yielded a residue which was digested a short time with 1 cc. of methyl alcohol and filtered. The undissolved portion was boiled with 7 cc. of water and filtered hot. The filtrate on cooling in ice gave 20 mg. of lysergic acid which melted at 238°.

$C_{16}H_{16}O_2N_2$.	Calculated.	C 71.69,	H 6.00,	N 10.45
	Found.	" 71.66,	" 6.07	
	"	" 71.82,	" 6.07	
	"			N 10.58
	"			" 10.63

For confirmation of its identity the methyl ester was prepared with diazomethane. After recrystallization from benzene it melted at 168°.

Lysergic Acid Hydrochloride—100 mg. of lysergic acid were dissolved in 4 cc. of dilute hydrochloric acid. After cooling the crystals which separated were collected with dilute HCl. When recrystallized from methyl alcohol the product melted with decomposition at 208–210°, depending somewhat on the rate of heating. It was dried for analysis at 120° and 2 mm.

$C_{16}H_{16}O_2N_2 \cdot HCl$.	Calculated.	C 63.03, H 5.63, N 9.19, Cl 11.62
	Found.	" 62.93, " 5.48, " 9.31, " 11.15

Lysergic Acid Sulfate—100 mg. of lysergic acid were dissolved in 8 cc. of hot water and a slight excess of dilute H_2SO_4 was added. Upon cooling the sulfate separated as leaflets. It was recrystallized from 6 cc. of hot water. It melts with decomposition at about 220°, depending somewhat upon the rate of heating. It was dried for analysis at 120° and 2 mm.

$(C_{16}H_{16}O_2N_2)_2 \cdot H_2SO_4$.	Calculated.	C 60.54, H 5.40, N 8.82
	Found.	" 60.97, " 5.47, " 8.76

Oxidation of Lysergic Acid with Nitric Acid—A number of experiments were performed but the following gave the best yield of the crystalline product.

400 mg. of lysergic acid were treated with 16 cc. of HNO_3 (sp. gr. 1.4) and the solution was placed on the steam bath for 20 hours. A clear red solution resulted, which was evaporated to dryness on the steam bath under reduced pressure. 10 cc. of water were added and the evaporation was repeated. This procedure was repeated twice in order to remove as much of the nitric acid as possible. The solid residue was boiled with 20 cc. of water and filtered after cooling. The filtrate was concentrated to approximately 1 cc. On long standing, leaflets gradually separated. The substance was collected with water. The yield was 10 to 12 mg.

The substance, which proved to be an acid, was rather slightly soluble in water. Recrystallization was accomplished by dissolving in a rather large volume of boiling water and concentrating to a small volume. It separates in small yellow rhombs which do not melt at 350°. The alkaline solution is of a brownish red color which again becomes pale yellow on acidifying.

In general properties it resembles closely the acid $C_{14}H_9O_8N$ obtained from ergotinine with the exception of the deep red color in alkaline solution.

$C_{13}H_8O_8N_2$.	Calculated.	C 48.75, H 2.51, N 8.75, NCH_3 9.05
	Found.	" 48.80, " 2.41
	"	" 48.80, " 2.70
	"	N 9.04
	"	NCH_3 7.94

Dihydrolysergic Acid—200 mg. of crystalline lysergic acid were suspended in 8 cc. of amyl alcohol and 400 mg. of sodium were added. The mixture was heated to the boiling point of the alcohol and shaken vigorously during the reduction. It was kept at this point until the sodium was dissolved. After cooling, 8 cc. of ether and then 4 cc. of water were added. The mixture was saturated with carbon dioxide. It was evaporated to dryness under reduced pressure and 10 cc. of ethyl alcohol were added. After reconcentration the residue was extracted with 15 cc. of hot alcohol. The alcoholic extract yielded a residue which was dissolved in 5 cc. of water. The solution was carefully treated with acetic acid which caused precipitation of dihydrolysergic acid. After collection with water 120 mg. of material were obtained which melted at 329°.

When recrystallized from water it melts with decomposition at approximately 336°, depending somewhat on the rate of heating. The dihydro acid is a more stable compound than lysergic acid and colors only slightly in the light. It is less soluble in water than lysergic acid.

$$[\alpha]_D^{20} = -88.0° \ (c = 0.5 \text{ in pyridine})$$

$C_{16}H_{18}O_2N_2$.	Calculated.	C 71.06, H 6.72, N 10.36
	Found.	" 71.24, " 6.68, " 9.78
	"	" 70.85, " 6.74

Dihydrolysergic Acid Methyl Ester—When an attempt was made to prepare the ester with diazomethane in the same way in which lysergic acid methyl ester was prepared, the yield was very poor. A considerable amount of an amorphous by-product was formed. Methyl alcoholic hydrochloric acid proved to be the better reagent.

50 mg. of dihydrolysergic acid were dissolved in 10 cc. of 4 per cent methyl alcoholic hydrogen chloride and allowed to stand at room temperature for 2 days. The solution was evaporated to dryness under reduced pressure on the steam bath and the residue was dissolved in 1 cc. of water. After precipitation with am-

monium hydroxide the solid was extracted with ether. The ether residue was recrystallized from benzene. The ester melts at 182°.

It is insoluble in water but soluble in the usual organic solvents. It crystallizes from benzene in broad leaves but can also be recrystallized from dilute alcohol.

$C_{17}H_{20}O_2N_2$.	Calculated.	C 71.78,	H 7.10,	N 9.85
	Found.	“ 71.58,	“ 7.03,	“ 9.78
	“	“ 71.81,	“ 6.84	

THE SYNTHESIS OF PENTOCYSTINE AND HOMOMETHIONINE

By VINCENT du VIGNEAUD, HELEN M. DYER, CHASE B. JONES, AND WILBUR I. PATTERSON

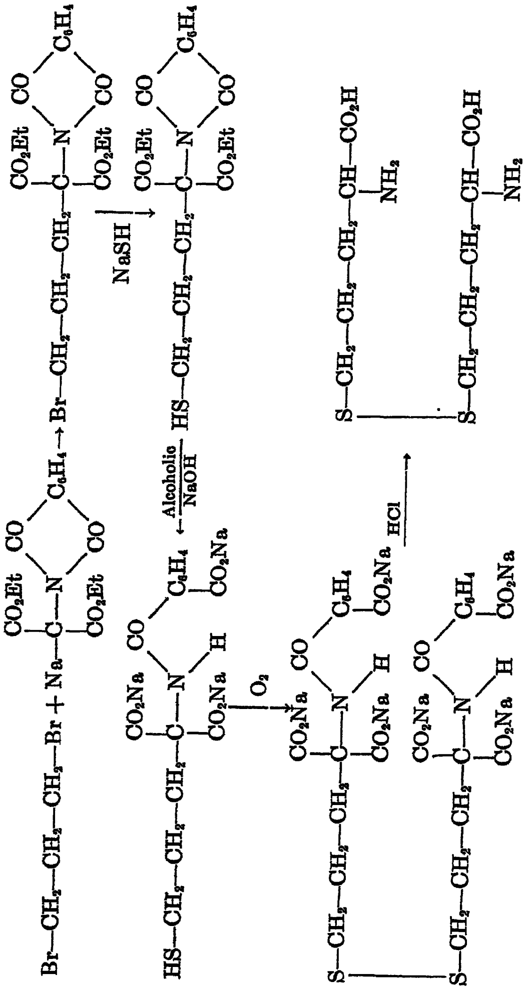
(From the Department of Biochemistry, School of Medicine, George Washington University, Washington)

(Received for publication, June 8, 1934)

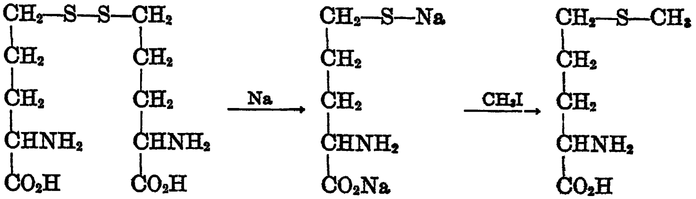
The evidence that homocystine, which was first encountered in a purely chemical way (1), is actually involved in metabolism as an intermediary step in the metabolism of methionine has been accumulating from various sources (2–6). Nevertheless, the question naturally arises as to whether the physiological behavior of homocystine such as its ability to support growth of animals on a cystine-deficient diet is non-specific in simply supplying sulfur in a utilizable form, or whether homocystine is more unique in bearing an actual metabolic relationship to methionine and possibly to cystine. If the former were true, then other disulfide amino acids might conceivably support growth. It was therefore thought desirable to synthesize homologues of cystine higher than homocystine in order that their action and fate in the body might be compared with that of cystine and homocystine. For reasons similar to those just mentioned, homologues of methionine were also desired for comparative studies.

The present communication deals with the synthesis of bis-δ-amino-δ-carboxybutyl disulfide which is the disulfide of the 5-carbon homologue of cysteine, and δ-methylthiol-α-aminovaleric acid which is the next higher homologue of methionine. For convenience we shall designate these compounds as pentocystine and homomethionine respectively.

The pentocystine was synthesized by means of the reactions shown in the accompanying series of equations.


NaSH
Alcoholic NaOH
O2
HCl

The homomethionine was synthesized from the pentocystine by reduction of the latter in liquid ammonia by metallic sodium and methylation of the pentocysteine so formed, as shown in the following equations.



$$\begin{array}{ll} CH_2\text{—}S\text{—}S\text{—} & CH_2 \\ | & | \\ CH_2 & CH_2 \\ | & | \\ CH_2 & CH_2 \\ | & | \\ CHNH_2 & CHNH_2 \\ | & | \\ CO_2H & CO_2H \end{array} \xrightarrow{Na} \begin{array}{l} CH_2\text{—}S\text{—}Na \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ CO_2Na \end{array} \xrightarrow{CH_3I} \begin{array}{l} CH_2\text{—}S\text{—}CH_3 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ CO_2H \end{array}$$

For confirmatory evidence of the structures of pentocystine and homomethionine various derivatives were prepared and analyzed.

EXPERIMENTAL

Preparation of Pentocystine—The diethyl-γ-bromopropylphthalimidomalonate was prepared by the condensation of diethyl sodium phthalimidomalonate and trimethylene bromide (7). 66 gm. of the former and 404 gm. of the latter were refluxed for 2 hours. The excess trimethylene bromide was removed by distillation *in vacuo*, the last portions being removed by steam distillation. About 360 gm. of the trimethylene bromide were recovered.

The replacement of the bromine atom by the sulfhydryl group was accomplished in the following manner. In a 1 liter round bottom flask 48 gm. of $Na_2S \cdot 9H_2O$ were dissolved in its own water of crystallization by heating the compound on a steam bath, and H_2S was then passed into the solution until the latter became acid to phenolphthalein. A solution of the diethylbromopropylphthalimidomalonate in 400 cc. of 95 per cent ethyl alcohol was then added to the solution of NaSH and the mixture was allowed to stand overnight. It was then refluxed for 1 hour on a steam bath after which the alcohol was removed by distillation *in vacuo*. The residue, dissolved in about 500 cc. of water, was transferred to a separatory funnel and layered with ether. Concentrated HCl was then added until the water layer was acid to Congo red and the solution was repeatedly extracted with ether. Considerable saponification of the ester no doubt occurred in the NaSH treatment since the ether extraction of the acid solution was found to increase materially the yield of the final product above that given by ether extraction of the original alkaline solution. The combined ether extracts were distilled to remove the ether and the remaining oil was saponified. The oil was dissolved in 100 cc. of 95 per cent alcohol, 200 cc. of 5 N NaOH were added, and the mixture was refluxed for 2 hours. The alkaline hydrolysis was carried out on the reduced form because it was expected that the sulfhydryl form would be more stable to alkali than the disulfide. However, the acid hydrolysis of the phthalamido compound, as will be described later, was carried out after oxidation of the sulfhydryl to disulfide. This was done to prevent possible lactone formation in the acid medium of the δ-thiol-α-aminovaleric acid which would have been formed from hydrolysis of the sulfhydryl intermediate.

To the above alkaline solution acid was added until the reaction was just alkaline to litmus. 2 drops of 5 per cent $FeCl_3 \cdot 6H_2O$ were added and the sulfhydryl compound oxidized by passing air through the solution until the nitroprusside test for the sulfhydryl group was negative. The solution was then made alkaline to phenolphthalein and filtered.

For hydrolysis of the phthalyl grouping and splitting out of CO_2, the filtered solution was diluted to 1 liter, 120 cc. of concentrated HCl were added, and the mixture was refluxed in an oil bath at 115–130° for 1½ hours. At this point 600 cc. more of concentrated HCl were added and the heating continued for another hour. The hydrolysate was then concentrated almost to dryness *in vacuo*. During the evaporation it was necessary to remove inorganic salt at intervals. In each instance the salt was thoroughly extracted with 20 per cent HCl and the extract returned to the distilling flask. The pentocystine hydrochloride was then extracted with approximately 0.5 N HCl. The pentocystine was then precipitated from the extract by careful neutralization to litmus with 5 N NaOH in the cold. The mixture after standing in an ice bath for an hour or two was filtered and the precipitate was washed with small portions of ice-cold water and finally with alcohol. The crude pentocystine was purified by treatment with carbex E in acid solution, followed by precipitation from the acid solution with alkali, and then finally by recrystallization from water. The yield of the final pure product, dried in a vacuum desiccator over P_2O_5, was 9 gm., or 30 per cent of the theoretical yield based on the amount of diethyl sodium phthalimidomalonate used. Further small quantities of the pure product can be obtained from the various mother liquors from the purification. The compound crystallizes in needle-like crystals and decomposes at 269–272° (corrected). The compound gives a negative sulfhydryl and positive disulfide test, a positive ninhydrin reaction, and a negative Sullivan test for cystine. The product for analysis was dried over P_2O_5 at 100° *in vacuo* and had the following composition.

0.0274 gm. substance: 0.0427 gm. $BaSO_4$ (Carius)
0.1000 " " : 6.30 cc. 0.1052 N HCl (Kjeldahl)
$C_{10}H_{20}O_4N_2S_2$. Calculated. N 9.45, S 21.64
Found. " 9.28, " 21.40

Preparation of S-Benzylpentocysteine—1 gm. of pentocystine was dissolved in about 20 cc. of liquid ammonia and reduced with metallic sodium as described by du Vigneaud, Audrieth, and Loring (8) for the reduction of cystine. To the liquid ammonia solution of the sodium salt of pentocysteine 1 cc. of benzyl chloride was added. After evaporation of the ammonia the residue was dissolved in 8 cc. of water and filtered. The filtrate was then neutralized with HCl and the precipitated S-benzylpentocysteine filtered. After being washed with methyl alcohol, the compound was recrystallized twice from water. 0.82 gm. of the final purified product was obtained. It melted with decomposition at 219–222° (corrected), and crystallized in rosettes of needle-like crystals. The compound was dried *in vacuo* at 100° over P_2O_5 and 0.1000 gm. of this material was analyzed for nitrogen by the Kjeldahl procedure. 5.75 per cent N was obtained, the calculated value for $C_{12}H_{17}O_2NS$ being 5.85 per cent N.

Preparation of Diformylpentocystine—1 gm. of pentocystine was dissolved in 15 cc. of 85 to 90 per cent formic acid. The solution was warmed to 60° and 5 cc. of acetic anhydride were added dropwise. After the solution had cooled to room temperature, 5 cc. of water were added to destroy any excess of acetic anhydride and the solution was concentrated *in vacuo* to about 3 cc. The formyl derivative slowly precipitated upon cooling. The compound was recrystallized from water. It is necessary to allow the solution to cool very gradually or otherwise an amorphous product is obtained. 0.6 gm. of purified product melting at 120–122° (corrected) was obtained. The compound for analysis was dried *in vacuo* at 61° over P_2O_5 and 3.541 mg. of the material were analyzed for nitrogen by the micro-Dumas method. The percentage of nitrogen obtained was 7.72 while the theoretical value was 7.95 per cent for $C_{10}H_{20}O_6N_2S_2$.

Preparation of Homomethionine and Formylhomomethionine—3 gm. of pentocystine were added to about 30 cc. of dry liquid ammonia in a 3-necked flask fitted with a mechanical stirrer and a calcium chloride tube. The flask was immersed in a cooling mixture of solid CO_2 and ether. Sodium was added to the flask in small portions until a very slight excess was indicated by the persistence of the blue color. To this solution 3, gm. of methyl iodide were slowly added. After removal of the excess ammonia, the residue was dissolved in 10 cc. of water and the solution neutralized with 10 per cent HI. The homomethionine began to crystallize and in order to complete the precipitation 4 volumes of ethyl alcohol were added. After standing in the ice box overnight the precipitate was filtered. The mother liquors and washings were concentrated to about 5 cc. and 4 volumes of alcohol were added. A further quantity of crystals was thus obtained. The products were recrystallized by dissolving them in a minimum of hot water and treating with 4 volumes of alcohol. The yield of the pure homomethionine was 2.7 gm. or 80 per cent of the theoretical yield. The compound melted at 272–274° (corrected). For analysis the compound was dried *in vacuo* over P_2O_5 at 100°. It had the following composition.

3.639 mg. substance: 0.263 cc. N at 24° and 772 mm.
0.956 gm. " : 0.1353 gm. $BaSO_4$ (Parr bomb)

$C_6H_{13}O_2NS$.	Calculated.	N 8.59, S 19.62
	Found.	" 8.63, " 19.44

The formyl derivative was prepared by slowly adding 2 cc. of acetic anhydride to a solution of 0.7 gm. of homomethionine in 6 cc. of 85 per cent formic acid. The reaction mixture was allowed to stand for 30 minutes and then evaporated to dryness *in vacuo*. The residue was extracted with 5 cc. of boiling ethyl acetate and the extract filtered while hot. Upon cooling 0.5 gm. of colorless prisms was obtained, melting at 122–123° (corrected). The compound was dried *in vacuo* at 61° over P_2O_5 and analyzed by the micro-Dumas method. The percentage nitrogen obtained was 7.44 while the theoretical value for $C_7H_{13}O_3NS$ was 7.32 per cent.

SUMMARY

The synthesis of the disulfide of the 5-carbon homologue of cysteine, bis-δ-amino-δ-carboxybutyl disulfide (pentocystine) and of the next higher homologue of methionine, δ-methylthiol-α-aminovaleric acid (homomethionine) has been presented.

Diformylpentocystine, S-benzylpentocysteine, and formylhomomethionine have also been prepared.

BIBLIOGRAPHY

1. Butz, L. W., and du Vigneaud, V., *J. Biol. Chem.*, **99**, 135 (1932-33).
2 du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).
3. Virtue, R. W., and Lewis, H. B., *J. Biol. Chem.*, **104**, 415 (1934).
4. du Vigneaud, V., Craft, H. A., and Loring, H. S., *J. Biol. Chem.*, **104**, 81 (1934).
5. Tarr, H. L. A., *Biochem. J.*, **28**, 192 (1934).
6. Pirie, N. W., *Biochem. J.*, **28**, 305 (1934).
7. Sörensen, S. P. L., *Z. physiol. Chem.*, **44**, 448 (1905).
8. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52**, 4500 (1930).

THE ANTITRYPSIN OF EGG WHITE*

By A. K. BALLS and T. L. SWENSON

(*From the Food Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington*)

(Received for publication, May 22, 1934)

The inhibitory action of egg white toward trypsin has been attributed in the main either to a specific enzyme inhibitor (1) or to some of the egg proteins which combine rapidly with the proteinase but are only slowly digested by it (2). The following experiments indicate that trypsin is inhibited by small quantities of a substance which may be extracted from eggs, that this substance is probably a protein hydrolysis product but not a true protein, and that it acts by displacing the enterokinase from its combination with the enzyme. The possibility of another type of tryptic inhibition, caused by the presence of difficultly digestible proteins, is not ruled out, but does not enter into this discussion.

In a recent paper Balls and Swenson (3) showed that the proteinase inhibitor of egg white was found in the thin or watery fraction of the white, whereas the thick white was definitely proteolytic. Since the thin white is formed during storage of the egg by the proteolysis of the thick white (3), it is reasonable to assume that the thick white contains the inhibitor in an inactive form, whereas the thin white, on the contrary, contains it in an active form which stops the unlimited breakdown of the egg proteins. It has been found possible to separate the inhibitory substance from the main bulk of the thin white by extracting the dried material with ammonia.

The antitryptic power of a preparation may be estimated, though only very roughly, by a method which determines the quantity of inhibitor needed to depress the activity of a standard trypsin to half its normal amount. The inhibitor was added to a fixed amount of practically inactive trypsin, and followed after 5 minutes by the amount of enterokinase which the enzyme should normally receive for complete activation. From Fig. 1, it appears that the relation between inhibition and amount of inhibitor follows a smooth curve, so that if several points are determined with different quantities of inhibitor, the quantity giving 50 per cent inhibition may be found by drawing the curve. Better methods are doubtless available, but this served to guide us to a procedure for concentrating the inhibitory substance.

---

* Food Research Division Contribution No. 216.

To insure a trypsin of constant quality, enough pig pancreas was dried with acetone and ether to last throughout this series of experiments. To prepare the enzyme a few gm. of the dried gland were extracted with 10 times its weight of 75 per cent glycerol. The extract was diluted with an equal volume of water, and adsorbed at neutral reaction three or four times with 0.1 its volume of alumina C_γ suspension (4) (10 cc. = 0.50 gm. of Al_2O_3). The adsorption removed small amounts of enterokinase and of the inhibitory substance, which may occur together with trypsin itself in pancreas, as Kunitz and Northrop (5) have stated. Tryptic activity was determined by the method of Willstätter *et al.* (6), with casein as a substrate. The details are shown in Table I. The preparation of the enterokinase has been described in a previous paper (3).

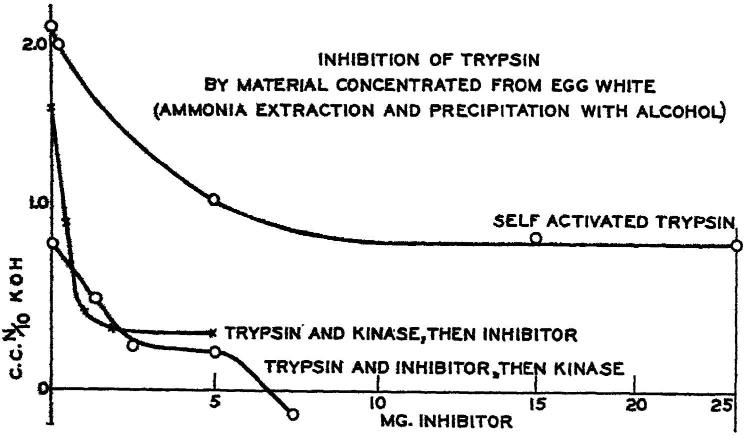


Fig. 1

Method of Concentrating Inhibitor—The thin white from several

TABLE I

*Progress in Concentration of Tryptic Inhibitor**

Preparation	Material tested	Amount producing 50 per cent inhibition	
		mg.	
A	Crude thin white	31.0	
B	Unheated ammonia extract (neutralized)	19.0	
C	Preparation B after heating	2.5	
D	Acetone-precipitated material from Preparation C	4.0	
E	Alcohol-precipitated material from acidified Preparation C	1.2	
F	Preparation E redissolved, clarified with acetic acid	0.9	
G	Preparation E redissolved, adsorbed (neutral) with $C\gamma$, elution by 0.04 N ammonia neutralized, precipitated by acetone	0.35	
H	Dialysate from solution of Preparation E (collodion, 48 hrs.)	0.20	
		Volume of preparation producing 50 per cent inhibition	Corresponding volume of original egg white represented
		cc.	*cc.*
A	Crude thin white	0.25	0.25
B	Extract with M/15 Na_2HPO_4	0.5	0.40
C	Phosphate extract of Preparation B heated, dialyzed 7 days	0.4	0.32

* Various amounts of dissolved inhibitor were mixed with 1.0 cc. of trypsin solution, followed in 5 minutes by 1.0 cc. of enterokinase (sufficient for full activation in the absence of inhibitor). Activation time was 30 minutes at 30°. Then 3 cc. of M ammonium chloride-ammonia buffer (pH = 9.1), 15 cc. of 6 per cent Hammarsten casein (dissolved in 0.06 N NaOH), and water up to 30 cc. were added. Titration of 10 cc. portions of the mixture at once and after 20 minutes at 30° measured the —COOH liberated by the tryptic digestion. Titration was made in hot alcohol, by the method of Willstätter *et al.* (6), with 0.1 N KOH. The inhibitory power is expressed as the weight of dry substance (or volume of solution) required to reduce the activity of the trypsin under the conditions given from its uninhibited value which was 1.2 cc. of 0.1 N KOH (for the titrated portion of 10 cc.) to 0.6 cc. of 0.1 N KOH.

eggs was dried with acetone and ether in the usual way, powdered, and stirred with 10 times its weight of 0.04 M ammonia for an hour. The alkaline liquid was poured off through a filter, and the insoluble matter washed with a little water. The extract was neutralized with M acetic acid, heated for 5 to 10 minutes at 75–80°, cooled, brought to pH 5.0 (approximately) with M acetic acid, and the precipitated protein centrifuged out. The inhibitory substance was then precipitated from the acid solution with 5 or 6 volumes of 95 per cent alcohol, thoroughly washed with alcohol, then with ether, and finally dried. About 1 gm. of material is obtained from 20 gm. of dried thin white.

Properties of Concentrated Substance

The substitution of alkaline phosphate for ammonia and of acetone for alcohol, and the removal of salts by dialysis did not materially affect the resulting product, from which it follows that the inhibitor is insoluble in acetone, ether, and alcohol, soluble in water, moderately heat-stable (continued boiling destroys it) and not dialyzable. While the inhibitor does not dialyze from impure solutions, where it is probably held by adsorption, it does dialyze slowly through collodion when present in purer form (Preparations E and F).

In water, the purified product (Preparation E) formed a faintly opalescent solution, which, after the addition of dilute acetic acid, was completely clarified by centrifugation without material loss. It gave positive biuret and Millon tests, but was negative to Molisch's test, Fehling's solution, and sodium nitroprusside. With alkaline lead solutions a considerable precipitate of lead sulfide was formed on warming. A solution of 10 mg. per cc. gave no visible precipitate with picric acid, trichloroacetic acid, tannic acid, or mercuric chloride. With phosphotungstic acid a heavy precipitate formed. The same behavior occurred on adding 2 volumes of saturated ammonium sulfate solution to the solution of inhibitor. However, much of the inhibitory substance remained in the solution, which still precipitated sulfide when warmed with lead and alkali.

The inhibitor is adsorbed by alumina C_γ at pH 5, and more readily from neutral solutions. Elution with 0.04 N ammonia appears to be reasonably complete. If acetone (5 volumes) is added to the neutralized elution, the precipitate contains about one-fourth of the original inhibitor in still higher concentration.[1]

The inhibitory power of a solution decreases slowly on standing in the cold. It is rapidly destroyed in hydrogen peroxide (0.01 N and 0.1 N) but not in 0.01 N iodine or in dilute sodium fluoride. Comparatively inert toward N H_2SO_4, it is completely destroyed by N NaOH.

The following analyses were made with Preparation E of Table I.

Total N
3.254 mg. substance: 0.297 cc. N_2 (Dumas) 27°, 770 mm. = 10.55
4.121 “ “ : 0.380 “ “ “ 27°, 771 “ = 10.65
Amino N
100 mg. substance: 0.98 cc. N_2 (Van Slyke) 24°, 764 mm. = 0.55
Total S[*]
8.610 mg. substance: 1.349 mg. $BaSO_4$ = 2.10
9.221 “ “ : 1.312 “ “ = 1.95
Titrations in Alcohol
100 mg. substance used 0.10 cc. 0.1 N HCl (brom-phenol blue)
100 “ “ “ 0.25 “ 0.1 “ KOH (phenolphthalein)
Optical Rotation
0.0290 gm. substance per cc. in 0.25 M NH_4OH; 20°; 5 cm. tube; $\alpha_D = -0.80^\circ$; $[\alpha]_D = -55^\circ$.

[*] Our thanks are expressed to Professor du Vigneaud of George Washington University for these analyses.

It is realized that the foregoing tests prove nothing definite about the composition of the substance, because the material is not crystalline and is evidently impure. They nevertheless indicate that the inhibitor is very probably neither a lipid nor a carbohydrate, but may possibly be an S—S-containing peptide of intermediate molecular weight. There is nothing which leads one to believe that the antitryptic agent of blood serum, which was identified by Jobling and Petersen (7) as unsaturated fatty acids, is related to the inhibitor prepared from egg white.

Summary of Tests Showing Effect of Several Reagents on Tryptic Inhibitor

The inhibitor used was precipitated by alcohol from the acidified ammonia extract of dried, thin white. After standing with the reagent, the treated inhibitor was mixed with 1 cc. of our standard trypsin preparation, then activated, and the activity of the trypsin determined as usual in a volume of 30 cc., of which 10 cc. portions were titrated with 0.1 N KOH. The titration results are given below as a direct measure of tryptic activity.

---

[1] The dialyzed preparation (Preparation H) is the most powerful we have made so far. It represented an increase of about 150 times over the original thin white, and still gave the sulfide test with alkaline lead solution.

Hydrogen Peroxide—10 mg. in 1 cc. of 0.01 N H_2O_2 for 30 minutes depressed the tryptic activity from 1.0 cc. to 0.6 cc. of 0.1 N KOH. The same volume of 0.01 N H_2O_2 alone had no effect on the trypsin, while the same quantity of inhibitor, untreated, depressed the activity from 1.0 cc. to 0.3 cc. A similar experiment with 0.05 N H_2O_2 showed that the H_2O_2 alone was still without effect on the trypsin, and the peroxide-treated inhibitor depressed the activity from 1.0 cc. only to 0.7 cc. Hydrogen peroxide evidently destroys the inhibitory effect.

Iodine in KI Solution—10 mg. of inhibitor per·cc. of 0.005 N iodine solution stood for 30 minutes, and showed no diminution of inhibitory power. Normal tryptic activity, 1.0 cc. of 0.1 N KOH; with added iodine solution only, 1.0 cc. of 0.1 N KOH; with iodine-treated inhibitor, 0.3 cc. of 0.1 N KOH; with untreated inhibitor, 0.3 cc. of 0.1 N KOH.

Hydrogen Sulfide—A solution of 10 mg. of inhibitor per cc., saturated with H_2S for 18 hours, was definitely acidified with acetic acid, H_2S removed in a vacuum, and the evaporated water restored. No change in inhibitory power was observed. 10 mg. inhibited 1 cc. of trypsin from 1.3 to 0.3 cc. both before and after this treatment.

Acid and Alkali—10 mg. were dissolved per cc. in N NaOH and in N H_2SO_4. Solutions stood 18 hours, then were neutralized. 20 mg. of alkali-treated inhibitor showed no inhibitory power whatever, while 20 mg. of acid-treated substance depressed trypsin from 1.2 to 0.3 cc.; 20 mg. of untreated inhibitor, from 1.2 to 0.1 cc. The substance resists strong acid very well, but its inhibitory effect is destroyed by strong alkali.

Sodium Fluoride—10 mg. of inhibitor were dissolved per cc. of water containing 1 mg. of NaF. After 30 minutes, 10 mg. depressed tryptic activity from 1.0 to 0.4 cc.; untreated inhibitor, from 1.0 to 0.3 cc.; sodium fluoride solution alone had no effect on trypsin.

Mode of Action of Inhibitor—The inhibitor does not combine with the active enzyme, thus preventing the access of a digestible substrate. This would constitute competitive inhibition, in which increasing amounts of inhibitor produce increasingly large effects. It follows from Fig. 1 that the reverse is true in this case. The inhibitor combines, therefore, either with the enterokinase or with the inactive enzyme.

Delezenne and Pozerski (1), who long ago studied the inhibition of trypsin by egg white, concluded that the inhibitor combines with the kinase because additional amounts of kinase decreased the inhibition. This we have repeated and can corroborate (Table II). Furthermore the reversal of inhibition by kinase is more marked the less inhibitor is present. On the other hand, we found that additional amounts of inactive enzyme have also the effect of removing the inhibition (Table II). The inhibition is therefore a reversible reaction. As Waldschmidt-Leitz (8) has shown that trypsin and kinase combine in definite proportions and that this reaction is to some extent reversible, our results and those of Delezenne and Pozerski can be explained equally well by assuming a combination of inhibitor either with inactive enzyme (Equation 1) or with kinase (Equation 2).[2] Willstätter, Bamann, and Rhodewald (9) also considered tryptic activity to depend upon a balance between activator and inhibiting factors. Combination of inhibitor with kinase does not necessarily imply that active trypsin is trypsin-kinase. Recently Dyckerhof, Miehler, and Tadsen (10) have advanced the idea that inactive trypsin is inhibited trypsin and that activation consists in combination of inhibitor with kinase, thus liberating the active enzyme (Equation 3). All three explanations are possible as long as the reaction is reversible.

(1) Trypsin-kinase + inhibitor $\rightleftarrows$ trypsin-inhibitor + kinase
(2) Trypsin-kinase + inhibitor $\rightleftarrows$ trypsin (inactive) + kinase-inhibitor
(3) Trypsin-inhibitor + kinase $\rightleftarrows$ trypsin (active) + kinase-inhibitor

---

[2] In an attempt to follow this question further, we succeeded in markedly inhibiting papain with the concentrate from egg white. Enterokinase added to inhibited papain did not reactivate the enzyme, but we found that inhibited trypsin could be reactivated by the addition of papain which was itself practically without action on the strongly alkaline casein solution at 30°. These results pointed definitely to a combination of trypsin or papain with the inhibitor, and not of inhibitor with enterokinase. Unfortunately, the results were obtained with one sample of papain only. In spite of earnest search, no other preparation of papain has been found which produced these effects in the slightest degree. It is necessary, therefore, to regard the question as still open.

TABLE II

*Reversal of Inhibition by Addition of Either Enterokinase or Inactive Trypsin**

Increasing amounts enterokinase on inhibited trypsin				Increasing amounts inactive trypsin on inhibited trypsin			
Active enzyme (diluted)	Inhibitor	Kinase	Tryptic activity (0.1 N KOH)	Active enzyme (diluted)	Inhibitor	Inactive enzyme (diluted)	Tryptic activity (0.1 N KOH)
cc.	*mg.*	*cc.*	*cc.*	*cc.*	*mg.*	*cc.*	*cc.*
2.5	0	0	1.4	0	0	1.0†	0.3
2.5	0	0.4	1.5‡	0	5	1.0†	0.2
2.5	5.0	0	0.8	2.5	0	0	1.4
2.5	5.0	0.1	1.0	2.5	0	0.5	1.3
2.5	5.0	0.4	1.0	2.5	0	1.0	1.4
2.5	5.0	1.0	1.2	2.5	0	2.5	1.4
2.5	5.0	2.5	1.4	2.5	5	0	0.8
				2.5	5	0.1	1.1
				2.5	5	0.5	1.1
				2.5	5	1.0	1.1
				2.5	5	2.5	1.4
				2.5	25.0	0	0.7
				2.5	25.0	0.1	0.7
				2.5	25.0	0.5	0.7
				2.5	25.0	1.0	0.9
				2.5	25.0	2.5	1.3

* Values of tryptic activity in Table II were obtained by digesting 5 cc. of 6 per cent casein (dissolved in 0.06 N NaOH), plus 1 cc. of M ammonia-ammonium chloride buffer, pH 9.1, plus enzyme and other additions to a total volume of approximately 10 cc. The entire solution was titrated in alcohol with 0.1 N KOH (alcoholic). The increase after 20 minutes at 30° over an exactly similar mixture titrated at once shows the —COOH formed by proteolysis. Active trypsin was prepared by diluting inactive glycerol extract of pancreas powder with 4 volumes of water, then incubating for 18 hours at 30°. Prokinase is thereby reduced to a minimum. Inactive trypsin was the same glycerol extract diluted with 1 volume of water and used at once. No activation time was allowed after addition of inactive trypsin or of kinase, except in controls where stated.

† Diluted with H_2O to 4 cc. for 30 minutes at 30°. All other mixtures, no activation time.

‡ 30 minutes (30°) after adding kinase. All other mixtures, no activation time.

Theoretically it should be possible to choose between these explanations by applying the law of mass action to the three cases. There are, however, technical difficulties to this, for the trypsin determinations may not be accurate enough to distinguish between the mathematical expressions involved. The probability that new trypsin and new kinase are formed, especially during activation, makes matters more complicated.

Behavior of Inactive Enzyme with Inhibitor—Much less inhibitor is required when it is added to the enzyme before, rather than after, the kinase. Total inhibition of the trypsin is easily obtained (Fig. 1). This is probably due to the fact that with most inactive trypsins, activation consists of far more than the simple combination of enzyme and kinase. Waldschmidt-Leitz and Harteneck (11) have shown that a prokinase may be present which on tryptic digestion changes to enterokinase, thus increasing the amount of activator beyond that intentionally added. Furthermore, Kunitz and Northrop (5) have recently isolated a protrypsin, a protein which on tryptic digestion produces the enzyme itself. There is, therefore, good reason to suppose that during the activation of our trypsin additional amounts of both trypsin and activator are formed. To produce practically complete inhibition, therefore, the amount of inhibitor, if added before activation, needs only to be sufficient to inhibit whatever trypsin was originally present.

Since the inhibition is reversible, there will always be a trace of active trypsin left, so that the inhibitor can only slow down the self-activation of the trypsin. Thus when inactive trypsin was diluted and incubated for 24 hours, the same result, a slightly inhibited enzyme, was obtained whether the inhibitor had been added before the incubation or, in the same amount, afterwards (Table III); yet no appreciable amount of this trypsin was activated when the fresh enzyme plus inhibitor was incubated, in the the usual way, for half an hour. This indicates that the action of the inhibitor is the same on the active and inactive enzyme preparations, but that the progress of the activation is retarded.

The existence of the inhibitor in trypsin preparations themselves can be shown by boiling the enzyme briefly, after which the preparations will often be found strongly inhibitory.

The effect of the inhibitor on inactive trypsin is not purely inhibitory, however. Usually a disappearance of carboxyl groups already present in the digestion mixture occurs. This is shown by the negative titration value in Fig. 1, and is one of many instances of this effect which we have observed with the inhibitor in all stages of purification.

Negative values for tryptic activity were obtained either by using very large quantities of inhibitor, or by adding inhibitor to the glycerol extract of pancreas before it was diluted with water (Table IV).

TABLE III

*Self-Activation of Trypsin with Insufficient Inhibitor**

Enzyme	Tryptic activity (0.1 N KOH)		
	No kinase	Excess kinase	+0.1 mg. inhibitor
	cc.	*cc.*	*cc.*
2.5 cc. freshly diluted	0.2	2.3	0.1
2.5 " diluted with water, then kept 24 hrs. at 30°	1.5	1.7	1.3
2.5 cc. diluted with water + 0.1 mg. inhibitor, then kept 24 hrs. at 30°	1.2	1.5	

* Tryptic activity was determined as in Table II. Enzyme used was glycerol extract of pancreas; 0.50 cc. diluted to 2.5 cc. with water. Quantities given are per titration portion of 10 cc.

TABLE IV

*Effect of Inhibitor on Inactive Enzyme**

	Tryptic activity after 30 min. at 30° with enterokinase (0.1 N KOH)			
Enterokinase, *cc.*	0	0.4	1.0	3.0
	cc.	*cc.*	*cc.*	*cc.*
Inhibitor (5 mg.) added to 2.5 cc. freshly diluted extract	+0.2			
Inhibitor (5 mg.) added to 0.5 cc. extract, then diluted to 2.5 cc.	−0.5	−0.3	+0.1	+0.4
No inhibitor	+0.3	+2.3		

* Tryptic activity in cc. of 0.1 N KOH, determined as in Table II. Enzyme used was freshly prepared glycerol extract of powdered pancreas.

These negative results prove nothing more than a disappearance of —COOH groups, but they may indicate some form of synthesis similar to that reported by various workers (12). We hope that by studying this protease inhibitor it may be possible to find out the nature of the reaction in which the —COOH disappears, whether the enzymic effect is a reversal of tryptic demolition or is due to a separate ferment, and what rôle the protease inhibitor plays in activating this process.

BIBLIOGRAPHY

1. Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, **55**, 935 (1903).
2. Hedin, S. G., *Z. physiol. Chem.*, **52**, 412 (1907).
3. Balls, A. K., and Swenson, T. L., *Ind. and Eng. Chem.*, **26**, 570 (1934).
4. Grassmann, W., Neue Methoden der Enzymforschung, Munich, 74 (1928).
5. Kunitz, M., and Northrop, J. H., *Science*, **78**, 558 (1933).
6. Willstätter, R., Waldschmidt-Leitz, E., Dunaiturria, S., and Künster, G., *Z. physiol. Chem.*, **161**, 191 (1926).
7. Jobling, J. W., and Petersen, W., *J. Exp. Med.*, **19**, 459 (1914).
8. Waldschmidt-Leitz, E., *Z. physiol. Chem.*, **132**, 181 (1924); **142**, 217 (1925).
9. Willstätter, R., Bamann, E., and Rhodewald, M., *Z. physiol. Chem.*, **188**, 109 (1930).
10. Dyckerhof, H., Miehler, H., and Tadsen, V., *Biochem. Z.*, **268**, 20 (1934).
11. Waldschmidt-Leitz, E., and Harteneck, A., *Z. physiol. Chem.*, **149**, 221 (1925). Pace, J., *Biochem. J.*, **26**, 1566 (1932).
12. Hedin, S. G., *Z. physiol. Chem.*, **207**, 213 (1932). Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, **62**, 15, 633, 675 (1924–25).

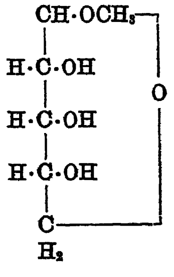
ACETONE DERIVATIVES OF *d*-RIBOSE. II.

By P. A. LEVENE and ERIC T. STILLER*

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 14, 1934)

The present work was undertaken with the purpose of preparing partially substituted ribose having one hydroxyl group, namely the one in position (2) or in position (4), unsubstituted. This substance was needed for the synthesis of the optically active 2- (or 4-) phosphoribitol.[1] It was expected that acetonylation of methylribopyranoside would lead to the desired derivative. From the figure below, it can be seen that acetone could be expected to enter the molecule of methylribopyranoside by condensing either in positions (2) and (3) or in (3) and (4).



Consequently, normal methylriboside was prepared by essentially the same method as that employed by Levene and Tipson.[2] Polarimetric observations of the initial glycoside formation in the cold showed that a maximum specific rotation of +7.7° was reached in 82 minutes and the final rotation was −57.3° (after boiling for 130 minutes). The difference in final specific rotation from that quoted by Levene and Tipson[2] is probably due to the fact that in the present investigations a specially purified sample of dry ribose was used, and that heating was continued longer.

---

* Commonwealth Fund Fellow.

[1] Levene, P. A., Harris, S. A., and Stiller, E. T., *J. Biol. Chem.*, **105**, 153 (1934).

[2] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **92**, 109 (1931).

The methylribopyranoside was condensed with acetone by the method previously described by the present authors.[3] In order to ascertain the structure of the condensation product, it was methylated by means of Purdie's reagents, yielding a monomethyl monoacetone methylriboside. On attempting to remove the acetone residue by means of 0.02 N hydrochloric acid—a reagent which should not effect the hydrolysis of the glycosidic methyoxyl group of a pyranoside[4]—the product reduced Fehling's solution strongly and was shown to contain 46 per cent of reducing monomethyl pentose. Further light was thrown on this unexpected result by the complete methylation of the hydrolyzed product, which gave a trimethyl methylriboside. The product on treatment with 0.02 N hydrochloric acid at 100° showed a fall in rotation and, by means of Willstätter titrations,[5] an increasing production of reducing material to a maximum of 52 per cent was demonstrated.

It is thus evident that during condensation of methylribopyranoside with acetone, a shift in the position of the lactal ring occurs, similar to that which has been shown by Levene and Meyer[6] to take place in the case of the condensation of methylmannopyranoside with acetone.

Confirmatory evidence of this partial ring shift was found in an examination of the action of *p*-toluenesulfonyl chloride on the acetone methylriboside. The product was shown to be a mixture of 5-*p*-toluenesulfonyl monoacetone methylribofuranoside with 4-*p*-toluenesulfonyl monoacetone methylribopyranoside. The former was compared with a specimen of known structure prepared directly from monoacetone methylribofuranoside and found to be identical. The latter was shown to be a pyranose derivative by its non-reaction with sodium iodide under standard conditions[7] and by the resistance of its glycosidic methoxyl group to hydrolysis under conditions which would cause hydrolysis of a furanoside.

---

[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933).

[4] Bott, H. G., Hirst, E. L., and Smith, J. A. B., *J. Chem. Soc.*, 658 (1930).

[5] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

[6] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **78**, 363 (1928).

[7] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

By treatment of the 5-*p*-toluenesulfonyl monoacetone methylribofuranoside with sodium iodide, an iodo derivative was obtained, conclusively demonstrating that the *p*-toluenesulfonyl residue was attached to the primary alcoholic group at position (5) of the ribose chain.

By hydrolysis of the 4-*p*-toluenesulfonyl monoacetone methylribopyranoside with aqueous methyl alcohol containing 5 per cent sulfuric acid in the cold, a 4-*p*-toluenesulfonyl methylribopyranoside was formed.

Thus it was definitely proved that on acetonylation methylribopyranoside in part rearranges into the furanoside. Indeed, study of a model reveals the fact that there is less strain in the structure of the 2,3-isopropylidene methylribofuranoside than in either of the two possible monoacetone derivatives of the methylpyranoside.[8]

EXPERIMENTAL

Preparation of Methylribopyranoside—The methylribopyranoside was prepared in essentially the same manner as described by Levene and Tipson.[2] Owing to the use of very specially purified ribose, the final rotation differed somewhat from that recorded by the above authors and by Levene, Raymond, and Dillon,[9] so a description of the preparation will be given.

20 gm. of finely powdered ribose (which had been twice recrystallized from absolute alcohol and dried to constant weight) were dissolved in 100 gm. of absolute methyl alcohol and 100 gm. of absolute methyl alcohol containing 3 gm. of dry hydrogen chloride were added.

The mixture was allowed to stand at room temperature while the specific rotation was followed polarimetrically. The specific rotation changed rapidly from −20.9° (6 minutes after admixture) and reached a minimum of +7.7° in 113 minutes, after which the rotation commenced to increase very slowly. After 113 minutes the solution was boiled gently in a water bath at 80°, polarimetric observations (see Table I) being made after the solution had been cooled and shaken with a little charcoal to remove the slight straw-yellow color. After boiling for 130 minutes, the specific rotation remained constant at −57.4°.

---

[8] The final purification of the tosyl derivatives was accomplished by Dr. R. S. Tipson. For this help as well as for his help in the preparation of the manuscript, we wish to express our thanks.

[9] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

The solution was now rendered neutral by addition of silver carbonate and the silver salts removed by filtration and thoroughly washed with small quantities of methyl alcohol. The combined filtrate and washings were evaporated to dryness under diminished pressure at a temperature below 35°, the last traces of solvent being removed under the high vacuum pump. The product (22.2 gm.) was a pale yellow, viscous syrup which did not reduce boiling Fehling's solution.

TABLE I

Condensation of d-Ribose with Methyl Alcohol in Presence of Hydrogen Chloride

	Time	$[\alpha]_D^{22}$		Time	$[\alpha]_D^{22}$
	min.	*degrees*		*min.*	*degrees*
Cold (27°)	0	−20.9	Cold (27°)	56	+6.5
	9	−16.8		58	+6.7
	13	−13.2		60	+6.8
	19	−7.8		65	+7.2
	27	−1.6		70	+7.4
	33	+1.4		76	+7.5
	35	+2.5		82	+7.7
	37	+3.1		113	+7.7
	39	+3.8		151	+7.0
	41	+4.0	Boiling (65°)	0	+7.7
	43	+4.7		25	−21.3
	45	+5.3		55	−39.3
	47	+5.4		75	−48.7
	49	+5.8		105	−55.9
	51	+6.0		130	−57.3
	53	+6.2		150	−57.3

$$[\alpha]_D^{25} = -46.5° \text{ (in water)}$$

In order to demonstrate that the amount of furanoside in the normal methylriboside was small, a weighed quantity was dissolved in 0.1 N hydrochloric acid and during 24 hours at 25° the specific rotation remained unchanged. The solution was then diluted with an equal volume of water, making the solution 0.05 N and samples were heated in sealed tubes in a boiling water bath. During the first half hour, the specific rotation remained constant (−46.2°) and thereafter fell very slowly (60 minutes, −43.0°).

Acetonylation of Methylribopyranoside—Methylribopyranoside (21 gm.) was dissolved in pure, dry acetone (400 cc.) containing 0.2 per cent sulfuric acid, and anhydrous copper sulfate (40 gm.) was added.[3] The mixture was shaken at 37° during 20 hours. The copper sulfate was now removed by filtration and thoroughly washed with small quantities of acetone. The combined filtrates and washings were shaken with calcium hydroxide until neutral and the calcium salts then filtered off and washed with acetone. The filtrate was evaporated to dryness under diminished pressure, the temperature being kept below 35°, and the final traces of solvent were removed under the high vacuum pump. The product was distilled under a high vacuum, giving a main fraction (19.1 gm.) boiling at 82–83° at 0.025 mm. This product was refractionated, giving 17.0 gm. of a pale yellow syrup which distilled at 84–86° at 0.05 mm. and had the following composition.

5.215 mg. substance: 5.670 mg. AgI

$C_8H_{13}O_4\ (OCH_3)$.	Calculated.	OCH_3 15.2
204.1	Found.	" 14.4

Preparation of Monomethyl Monoacetone Methylriboside—Monoacetone methylriboside (3.9 gm.) was methylated by means of Purdie's reagents (20 cc. of methyl iodide and 16 gm. of silver oxide) with vigorous stirring. The product was isolated in the usual way and was distilled under a high vacuum, giving 3.5 gm. of a colorless mobile syrup (b.p. 68–69° at 0.04 mm.) which had the following composition.

5.604 mg. substance: 11.570 mg. AgI

$C_8H_{12}O_3\ (OCH_3)_2$.	Calculated.	OCH_3 28.5
218.1	Found.	" 27.3

Hydrolysis of Monomethyl Monoacetone Methylriboside—The product (3.5 gm.) was dissolved in 44 cc. of 0.02 N hydrochloric acid and heated at 100°. Polarimetric observations showed a rapid change in rotation, from an initial value of −64.5° to a constant value of −22.0° after 30 minutes. The solution was rendered neutral with barium carbonate and the water removed under diminished pressure at a temperature below 30°. The product was mixed with benzene and the solvent distilled off under diminished pressure in order to remove the last traces of water. This treatment was repeated three times. The product, 2.5 gm. (theory 2.6 gm.), was a pale yellow viscous syrup which was extracted by means of absolute alcohol. It reduced warm Fehling's solution strongly.

The amount of reducing material present was estimated by the method of Willstätter and Schudel[5] and shown to be 46.5 per cent.

Exhaustive Methylation of Hydrolytic Product—The product from the acid hydrolysis (2.3 gm.) was dissolved in 25 cc. of methyl alcohol containing 1.5 per cent dry hydrogen chloride and allowed to stand at room temperature until the rotation became constant. In 1 hour a constant specific rotation of −41.9° was attained. The solution was rendered neutral with silver carbonate and the silver salts removed by filtration and thoroughly washed. The combined filtrate and washings were evaporated to dryness under diminished pressure at a temperature below 30°.

The product (2.4 gm.) was methylated by means of Purdie's reagent (30 cc. of MeI, 20 gm. of Ag_2O) with vigorous stirring. On isolation in the usual manner, the syrupy product was remethylated with half the above quantities of Purdie's reagent.

The product was isolated and distilled at 58–60° at 0.03 mm. The n_D^{27} was 1.4445, and the composition as follows:

3.555 mg. substance: 6.820 mg. CO_2 and 2.830 mg. H_2O
4.805 " " : 21.888 " AgI
$C_5H_6O\,(OCH_3)_4$. Calculated. C 52.4, H 8.8, OCH_3 60.2
206.1 Found. " 52.3, " 8.9, " 60.2

Partial Hydrolysis of Mixed Trimethyl Methylribosides—369.0 mg. of the fully methylated product were dissolved in 25 cc. of 0.02 N hydrochloric acid. 4 cc. samples were sealed in tubes and heated at 98°. At various intervals of time, a tube was removed from the heating bath and quickly chilled in ice. The polarimetric observations were performed for mercury green light (λ = 5781) and the amount of reducing sugar present in each sample was determined by means of a Willstätter-Schudel titration (see Table II).

Preparation of 5-p-Toluenesulfonyl 2,3-Monoacetone Methylribo-

furanoside—2,3-Monoacetone methylribofuranoside was prepared by the method previously described by Levene and Stiller[10] by treating ribose with acetone containing 5 per cent of methyl alcohol in the presence of 0.2 per cent sulfuric acid and anhydrous copper sulfate.

2.8 gm. of monoacetone methylribofuranoside were dissolved in the minimum quantity of dry pyridine, *p*-toluenesulfonyl chloride (3.0 gm.) was added, and the mixture was allowed to stand at room temperature overnight. 1 drop of water was added and the solution (cooled in ice) was allowed to stand for 30 minutes.

Chloroform and water were now added and the aqueous layer was extracted three times with small quantities of chloroform. The combined extracts were washed with dilute sulfuric acid, dilute sodium hydroxide, and finally, with water. After drying over anhydrous sodium sulfate and filtering, the filtrate was evaporated under reduced pressure, giving 5.0 gm. of pale yellow, viscous syrup which crystallized rapidly on scratching under a little dry ether. After recrystallization from absolute alcohol the fine white needles had a melting point of 83–84°. The substance had the following composition.

TABLE II

Hydrolysis of Mixed Trimethyl Methylribosides

Time	$[\alpha]_{5781}$	Reducing sugar
hrs.	*degrees*	*per cent*
0	−39.41	
1	−35.40	40.7
2	−33.25	47.1
3	−33.79	52.2

4.401 mg. substance: 8.690 mg. CO_2 and 2.370 mg. H_2O
12.265 " " : 7.685 " $BaSO_4$
8.442 " " : 5.520 " AgI
$C_{16}H_{22}O_7S$. Calculated. C 53.6, H 6.2, S 8.9, OCH_3 8.7
358.2 Found. " 53.8, " 6.0, " 8.7, " 8.6

The specific rotation of the *p*-toluenesulfonyl derivative was

$$[\alpha]_D^{25} = \frac{-0.74° \times 100}{2 \times 1.043} = -35.5° \text{ (in absolute ethyl alcohol)}$$

---

[10] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

Action of Sodium Iodide on 5-p-Toluenesulfonyl Monoacetone Methylribofuranoside—The 5-*p*-toluenesulfonyl monoacetone methylribofuranoside (1 gm.) was treated in acetone with sodium iodide (1 gm.) in a sealed tube at 100° for 2 hours. The solution was diluted with water and extracted repeatedly with chloroform. The chloroform extracts were washed with sodium thiosulfate solution in order to remove any free iodine and then dried over anhydrous sodium sulfate. On removal of the solvent, a pale yellow syrup (1 gm.) (which resisted all attempts at crystallization) remained.

It had the following composition.

7.595 mg. substance: 5.680 mg. AgI
10.165 " " : 7.535 " "
$C_8H_{15}O_4I$. Calculated. I 40.4, OCH_3 9.9
314.1 Found. " 40.4, " 9.8

Preparation of 4-p-Toluenesulfonyl Monoacetone Methylribopyranoside—The mixture of monoacetone methylribosides described above (5 gm.) was treated with 1.1 moles of *p*-toluenesulfonyl chloride in dry pyridine and allowed to stand overnight. The product was isolated as described above and 11.2 gm. of substance were isolated, crystallizing in white needles from absolute alcohol. The product began to soften at 78° and had melted completely at 127°.

By fractional crystallization from absolute alcohol, two homogeneous fractions were isolated. The lower melting fraction was identical with the 5-*p*-toluenesulfonyl monoacetone methylribofuranoside described above, m.p. 84–86°, giving no depression on admixture and forming an iodo derivative which did not crystallize.

The other pure fraction, m.p. 144–145°, had the following composition.

4.495 mg. substance: 8.870 mg. CO_2 and 2.530 mg. H_2O.
11.700 " " : 7.610 " $BaSO_4$
6.511 " " : 4.350 " AgI
$C_{16}H_{22}O_7S$. Calculated. C 53.6, H 6.2, S 8.9, OCH_3 8.7
358.2 Found. " 53.8, " 6.3, " 8.9, " 8.8

It showed the following specific rotation.

$$[\alpha]_D^{25} = \frac{-1.89° \times 100}{2 \times 0.822} = -114.9° \text{ (in absolute ethyl alcohol)}$$

This fraction of higher melting point did not react with sodium iodide, the starting material being recovered unchanged after treatment under standard conditions for 2 hours.

The main portion of the original mixture of *p*-toluenesulfonyl derivatives, when heated with sodium iodide in acetone at 100° in a sealed tube for 2 hours, gave a product which partly crystallized. The crystals, on separation from the syrupy iodo compound identical with that already described, proved to be 4-*p*-toluenesulfonyl monoacetone methylribofuranoside (m.p. 144–145°).

Hydrolysis of Acetone Residue from 4-p-Toluenesulfonyl Monoacetone Methylribopyranoside—4-*p*-Toluenesulfonyl monoacetone methylribopyranoside (2.5 gm.) was dissolved in 100 cc. of methyl alcohol and 20 cc. of 5 per cent sulfuric acid were added. A crystalline precipitate formed and on allowing the mixture to stand at room temperature for 4 hours, the suspension disappeared. 20 cc. of 5 per cent acid were then added and, after the precipitate had dissolved (4 hours), further addition of acid produced no precipitate. The acid was neutralized with barium carbonate and the barium salts removed by filtration. On removing the solvent under reduced pressure, the product crystallized spontaneously in white needles. It was recrystallized from ether, the substance having a melting point of 124° and the following composition.

4.221 mg. substance: 7.610 mg. CO_2 and 2.190 mg. H_2O
8.244 " " : 6.031 " $BaSO_4$
6.752 " " : 5.091 " AgI
$C_{13}H_{18}O_7S$. Calculated. C 49.0, H 5.7, S 10.1, OCH_3 9.8
318.2 Found. " 49.2, " 5.8, " 10.1, " 9.9

Its specific rotation was as follows:

$$[\alpha]_D^{27} = \frac{-0.82° \times 100}{2 \times 1.024} = -40.0° \text{ (in chloroform)}$$

IMP. INST. AGR. RES. PUSA
25. OCT. 1934

VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS

IV. REPRODUCTION AND LACTATION UPON FAT-FREE DIETS*

By HERBERT M. EVANS, SAMUEL LEPKOVSKY, and ELIZABETH A. MURPHY

(From the Institute of Experimental Biology, University of California, Berkeley)

(Received for publication, March 20, 1934)

INTRODUCTION

Studies with diets devoid of the essential unsaturated fatty acids but with all other known dietary essentials are of recent origin (1–3), and it is consequently too early to expect thorough treatment of special aspects of the physiology of such animals, for instance their reproduction and lactation. An excellent beginning was made by Burr and Burr (4) whose initial results, though based upon only three rats, clearly indicated abnormality or failure in reproduction. They also made a beginning in the study of lactation. Lard fatty acids were fed to females, making possible normal young; and four litters totaling twenty-five young were successfully suckled without mortality with an average weight at weaning of 29 gm.

The reproduction and lactation of rats on fat-free diets has been thoroughly investigated with large numbers of rats and forms the subject of this report.

EXPERIMENTAL

Technique for Gestation Studies—The diet used (Diet 667) consisted of fat-free casein[1] L-XI, 24.0; sucrose, 72.1; and Salt Mixture 185 (5), 3.9. The vitamin B and vitamin G requirements were satisfied by the separate daily feeding of 1 gm. of ether-extracted brewers' yeast,[2] the vitamin A and vitamin D requirements by the daily feeding of the non-saponifiable matter from 83 mg. of cod liver oil[3] (6), and the vitamin E requirement by the weekly feeding of the non-saponifiable matter from 500 mg. of wheat germ oil (3).

---

* Aided by grants from the Research Board and the College of Agriculture of the University of California, and the Rockefeller Foundation.

[1] We are grateful to the Golden State Company, Ltd., for its generosity in giving us this casein.

The rats were maintained on the fat-free diets for about 5 months, when some were fed the essential unsaturated fatty acids as 10 mg. of walnut oil. When the rats were from 6 to 7 months old, the walnut oil was replaced with 50 mg. of a preparation rich in the essential unsaturated fatty acids (Preparation 48-e). The rats receiving this supplement, in all 52, were bred and yielded reproduction data with the essential unsaturated fatty acids. 59 rats were kept without addition of the essential unsaturated fatty acids and were mated when 6 to 7 months old, yielding the reproduction data without the essential unsaturated fatty acids. After the first gestation some of these rats were used to determine the possible importance of increased amounts of vitamins A, D, E, and carotene upon the course of gestation upon fat-free diets.

Whenever estrus occurred, the females were placed in cages with wire screen bottoms with males which had been maintained on a natural food diet. After mating occurred the vaginal contents were studied daily and the course of the gestation observed. The animals were weighed every 5 days and daily from the 20th day of gestation to the time of littering or to the 25th day in case a litter was not cast.

Technique for Lactation Studies—We encountered difficulty in rearing young in cages with wire mesh bottoms so that the method was adopted of removing the wire mesh bottoms at the 20th day and using wood shavings throughout the lactation period. Daily observations of the condition of the mother and young were made and both were weighed every 5 days. The known vitamins were all increased in order to make sure that the fat-free diet was fully adequate for lactation in all the known dietary essentials.

---

[2] We wish to thank President R. M. Allen of the Vitamin Food Company, Inc., of New York for generously supplying a whole dried brewers' yeast remarkably high in its content of the antineuritic vitamin B.

[3] We wish to thank Mead Johnson and Company for generously supplying the cod liver oil.

Preparation of Materials Used—Casein was washed with acidulated water (7) and extracted for 1 week with ether.

A reliable preparation of the essential unsaturated fatty acids (Preparation 48-e) was made from corn oil by the crystallization and removal of its insoluble lithium salts from 80 per cent ethyl alcohol. It contains approximately 80 per cent linoleic acid and 20 per cent oleic acid.

The vitamin B concentrate (Preparation 9-G) was prepared by extracting rice polish with 25 per cent alcohol, concentrating *in vacuo*, and adding 95 per cent alcohol to the syrup to a concentration of 80 per cent. It is filtered, and the filtrate concentrated *in vacuo* so that 1 cc. = 10 gm. of rice polish.

The vitamin G concentrate (Preparation 53) was prepared by extracting hog livers with boiling water, concentrating *in vacuo*, and removing the glycogen and other substances with 2 volumes of alcohol, and finally concentrating so that 1 cc. = 10 gm. of fresh liver.

Results of Studies on Gestation

Gestation without Essential Unsaturated Fatty Acids—Normal reproduction without the essential unsaturated fatty acids is impossible (Table I). About 20 per cent of the females failed to litter after implantation had occurred as indicated by the finding of red blood cells in the vaginal tract from the 12th to the 20th day. 6 per cent of the pregnant females died during littering, and in 95 per cent of the pregnancies there were peculiar[4] prolonged gestation periods, from the 23rd to 25th day. 80 per cent of the young were born dead and the remainder died soon after birth. On an average, only three young were born per litter, and had an average weight of only 4.0 gm.

Influence of Increased Amounts of Vitamins A, D, E, and of

---

[4] Apparently there is a derangement of the birth mechanism necessary to expel the young on the normal day of parturition. Littering is often accomplished only with great difficulty, 24 hours being sometimes consumed in the process, and frequently accompanied with such great weakness of the mother as to render its survival uncertain. Autopsy of two rats on the 19th day of gestation and two rats on the 23rd day disclosed macerated young, and some resorption sites. The corpora lutea of the mothers very frequently had an unnatural, white, chalky appearance. Copious hemorrhage was sometimes observed.

TABLE I

Reproduction Data upon Fat-Free Diet 667 with and without Essential Unsaturated Fatty Acids

Females between 5 and 8 months old were used; essential unsaturated fatty acids were fed when the rats were 6 to 7 months of age.

	Diet 667	Diet 667 + essential unsaturated fatty acids
Total No. of females in group	59	52
No. of females placed at least once with male	47	51
Total instances of estrous female placed with male	97	122
No. of matings resulting	51	78
Per cent of matings	52.6	64.0
No. of matings showing sign of implantation	48	67
Per cent of matings showing sign of implantation	93.8	85.9
No. of instances with implantation sign; no litter resulted	9	4
Per cent of failures to litter after implantation sign	18.6	5.9
No. of animals on which autopsy was performed before littering	2	2
No. of animals dying during littering	3	2
" " litters	35	59
Per cent of matings resulting in litter†	69.0	76.0
Average no. of young per litter	3.0	5.8
No. of young born dead	85	49
Per cent of young born dead	80.0	14.4
No. of young born living	21	296
Per cent of young born living	20.0	85.6
Average weight of young at birth, *gm.*	4.0	5.0
" " " mothers at time of littering, *gm.*	185	202
Per cent of animals having 22 day gestation period	4.2	59.3
Per cent of animals having 23 day gestation period	29.3	37.0
Per cent of animals having 24 day gestation period	66.5	3.5
Per cent of animals having 25 day gestation period	0	0
No. of mothers suckling young	0	31
Per cent of mothers suckling young	0	52.6

Carotene upon Course of Gestation When Fat-Free Diet 667 without Essential Unsaturated Fatty Acids Is Fed (Table II)—The occasional failure to litter after implantation had occurred, a phenomenon characterizing the lack of vitamin E, led us to suspect possible inadequacy of vitamin E. The vitamin E was accordingly trebled by feeding the non-saponifiable matter from 1.5 gm. of wheat germ oil per week in the case of four animals. One of these continued to show the same phenomenon—failure to litter after the implantation sign—and the other three had prolonged gestation periods similar to most animals on the fat-free diet.

TABLE II

Reproduction Data on Rats Fed Fat-Free Diet 667 without Essential Unsaturated Fatty Acids

At time of mating, additional supplements were added as noted. All rats had previously had one poor litter.

	Vitamin E trebled	1 mg. carotene daily	Vitamins A and D trebled
No. of females in group	4	4	4
" showing implantation sign	4	4	4
" " " " and failing to litter	1	2	2*
No. of litters	3	2	2
Average No. of young per litter	3.0	5.5	2.0
No. of young born dead	7	6	4
Per cent of young born dead	77.5	55.0	100.0
No. of young born living	2	5	0
Per cent of young born living	22.5	45.0	0
Average weight of young at birth, *gm.*	4.0	4.8	0
" " " mothers at time of littering, *gm.*	184	186	178
No. of animals having 22 day gestation period	0	1	0
" " " " 23 " " "	2	0	2
" " " " 24 " " "	0	0	0
" " " " 25 " " "	1	1	0

* Autopsy was performed on rats on 23rd day; no young found in one and one macerated young found in the other.

Vitamins A and D were also increased by trebling the daily feeding of the non-saponifiable matter from cod liver oil. Again one of the four animals failed to litter after having shown the implantation sign, and the other three followed the usual course of gestation on the fat-free diet.

Because of the high state of unsaturation of carotene, the possibility existed that it might exert a beneficial effect on the fat-free diet in the absence of the essential unsaturated fatty acids. Accordingly, 1 mg. of carotene was fed daily to four animals. Two of them failed to litter after implantation and the other two behaved no differently from the animals on the fat-free diet.

A summary of results of gestation without the essential unsaturated fatty acids follows. (1) About 95 per cent of the animals littering do so from 1 to 3 days late and 80 per cent of the young are born dead. (2) Both the number and weights at birth of the young are markedly subnormal. (3) In about 20 per cent of the cases there is failure to litter after implantation occurs, but inadequate vitamin E is not the cause of these failures. (4) Vitamins A and D are not limiting factors. (5) The unsaturated compound, carotene, did not replace the essential unsaturated fatty acids. (6) Suckling did not occur.

Gestation with Essential Unsaturated Fatty Acids—The addition of the essential unsaturated fatty acids (Preparation 48-e) markedly affected the course of gestation (Table I). In every respect there were astonishing improvements. The average number of young born per litter was increased from 3 to 5.8 and the average weight from 4 to 5 gm. Only 14 per cent of the young were born dead as compared with 80 per cent when the essential unsaturated fatty acids were withheld. There were few prolonged gestation periods, 37 per cent littering on the 23rd day, and only 4 per cent on the 24th day. 52 per cent of the pregnant females suckled their young as compared with none when the diet was not supplemented with the essential unsaturated fatty acids.

Failures to litter were reduced from 20 to 6 per cent. Some allowance should be made for these, since all the animals did not receive the essential unsaturated fatty acids for the same length of time, but were maintained on the fat-free diet until, in many cases, characteristic deficiencies due to the absence of the essential unsaturated fatty acids set in. Such rats may have been so injured that the length of time the essential unsaturated fatty acids were administered was not sufficient to restore them fully to normal.

A summary of the results obtained in gestation when the essential unsaturated fatty acids were fed follows. (1) The characteristic prolonged gestation periods were greatly reduced. (2) The number of young per litter and their average weights at birth were definitely increased. (3) There was a marked reduction in the number of animals failing to litter. (4) The weight of the mothers at littering was greater, and the majority of them nursed their young.

Lactation

Results with Fat-Free Diet with Increased Amounts of All Known Vitamins—All the animals used for our lactation studies reported in this communication had received the active preparation of the essential unsaturated fatty acids (Preparation 48-e) before and during gestation. To eliminate any possible deficiency in the known factors during lactation, it seemed advisable to increase all the known accessory factors. The vitamin B supplement was increased by the daily addition of 0.8 cc. of Preparation 9-G, and the vitamin G supplement by the daily addition of 0.5 cc. of autoclaved liver extract (Preparation 53). The vitamin F supplement (the essential unsaturated fatty acids) was increased by raising the daily feeding of Preparation 48-e from 50 to 150 mg. The vitamin E supplement was increased by weekly feeding the non-saponifiable matter from 1.5 gm. of wheat germ oil instead of that from 0.5 gm. of the oil. Vitamins A and D were increased by the daily feeding of the non-saponifiable matter from 249 mg. of cod liver oil instead of that from 83 mg. of the oil.

The mortality of the young in this experiment was very high (Table III), only 51.6 per cent of the young being weaned, with an average weight at weaning of 28.8 gm. The mothers gained on an average about 11 gm. during lactation.

Results with Diet I (8) Plus Lettuce—When rats were changed to our stock Diet I on the day of littering, the results were quite different. The mortality was still quite high, 31 per cent dying before weaning time, but there was a distinct improvement in the weights of the young at weaning. The average weight was 34.5 gm., which is more nearly the weight of normal young at that age. The mothers fared well, gaining an average weight of 24 gm. during lactation.

Results with Diets of Purified Ingredients Containing 25 Per Cent Lard or Butter Fat[5] *with All Supplements Increased*—When diets containing 25 per cent of lard or butter fat with all the supplements in increased amounts were fed, the lactation performance was as good as that encountered on our stock Diet I (Table III).

TABLE III

Lactation on Purified Diets to Which Were Added Increased Amounts of All Known Vitamins, Including Essential Unsaturated Fatty Acids (Vitamin F); Effect of Stock Diet I, 25 Per Cent Lard, and 25 Per Cent Butter Fat

	Diet 667+ increased amounts of vitamins A, B, D, E, F, G	Diet I	Diet 667 + 25 per cent lard + increased amounts of vitamins A, B, D, E, F, G	Diet 667 + 25 per cent butter fat + increased amounts of vitamins A, B, D, E, F, G
No. of females	6	3	6	4
" " young	31	16	36	23
Young dead before 5th day	11	2	3	3
" " after " "	4*	3	0	1
Average No. per litter	5	5	6	5.5
" weight at birth, *gm.*	4.9	4.9	5.6	5.4
" " on 5th day	6.4	6.7	9.3	8.3
" " " 10th "	11.8	12.3	16.4	18.0
" " " 15th "	18.4	21.7	26.3	22.9
" " at weaning	28.8	34.5	39.3	33.6
Total gain in weight, *per cent*	587	704	701	622
No. weaned	16	11	33	19
Per cent weaned	51.6	68.7	91.6	82.5
Av. wt. of mothers at littering, *gm.*	205	234	224	215
" " " " " weaning, "	216	262	238	229
No. of mothers weaning young	6	3	6	4
Average gain in weight of mothers, *gm.*	11	24	13	14

* Two were killed accidentally.

In both cases, the mortality of the young was definitely decreased. There was also great improvement in the weights of the young at weaning, an average of 39.3 gm. being attained by the young of mothers receiving the diet containing lard, and 33.6 gm. by the young of mothers receiving the diet containing butter fat. On both diets the mothers made satisfactory gains during lactation. An apparent superiority of the diet containing lard was evidenced by the unusually vigorous young, their weights at littering averaging 5.6 gm., the highest recorded in these experiments.

---

[5] These diets contain casein L-XI, 30; sucrose, 41; lard or butter fat, 25; Salt Mixture 185 (5), 4.

A summary of the results of lactation follows. (1) We may be permitted to disregard the high mortality in suckling young, since it continued to occur when mothers were shifted to an excellent diet of natural foodstuffs (Diet I) and is hence to a great extent attributable to malcondition of the young born from mothers reared on the fat-free diets. But poor lactation performance is portrayed by the poor weights of the young at weaning, since these are markedly improved by shift in the maternal diet. Lactation was unsatisfactory on fat-free diets, regardless of the addition of increased amounts of all the known vitamins. (2) When the above diets contained 25 per cent butter fat or lard, increase in the weights at weaning to 33 gm. or more resulted. It appears therefore that appreciable amounts of certain fats are necessary for normal lactation in addition to need for the essential unsaturated fatty acids. (3) A diet of natural foodstuffs (Diet I) did not improve the weights at weaning beyond the improvement secured by the addition of 25 per cent butter fat or lard to our basic diet.

SUMMARY

1. Successful gestation upon the fat-free diet was not possible. There occurred a characteristic prolongation of gestation for from 1 to 3 days, together with impairment of the birth mechanism. Small litters of undersized young were born dead or so weak that they died soon after birth. In a fifth of the cases there was failure to litter after positive placental sign (resorption). Some maternal mortality was encountered. Fortification with carotene, or increased amounts of vitamins A, D, or E, did not essentially modify the outcome.

2. The addition of the essential unsaturated fatty acids resulted in marked improvement in all the abnormalities indicated.

3. When all the known vitamins were added in increased amounts, lactation on the fat-free diet plus the essential unsaturated fatty acids was possible, but not highly successful, the weights of the young at weaning being still subnormal. Inclusion of 25 per

cent lard or butter fat, however, gave weights at weaning approaching normal, and diets of natural foodstuff could not confer greater improvement.

BIBLIOGRAPHY

1. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **82**, 345 (1929).
2. McAmis, A. J., Anderson, W. E., and Mendel, L. B., *J. Biol. Chem.*, **82**, 247 (1929).
3. Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, **96**, 143 (1932).
4. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **86**, 587 (1930).
5. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
6. Steenbock, H., Sell, M. T., and Buell, M. V., *J. Biol. Chem.*, **47**, 89 (1921).
7. Evans, H. M., and Lepkovsky, S., *J. Nutrition*, **3**, 353 (1930–31).
8. Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, **92**, 615 (1931).

VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS

V. REPRODUCTION AND LACTATION UPON DIETS CONTAINING SATURATED FATTY ACIDS AS THEIR SOLE SOURCE OF ENERGY*

BY HERBERT M. EVANS, SAMUEL LEPKOVSKY, AND ELIZABETH A. MURPHY

(*From the Institute of Experimental Biology, University of California, Berkeley*)

(Received for publication, March 20, 1934)

Reproduction and lactation upon fat-free diets and the rôle played by the essential unsaturated fatty acids have been reported in detail (1). The rôle of these essential unsaturated fatty acids in reproduction and lactation with diets whose sole source of energy consists of saturated fatty acids forms the subject of the investigation reported in this paper. Diet 675 used in this study consisted of casein L-XI (1), 40; hydrogenated coconut oil,[1] 60; and Salt Mixture 185 (2), 5. The usual vitamin supplements for fat-free diets were furnished by 1 gm. of ether-extracted brewers' yeast[2] daily for vitamins B and G, non-saponifiable matter from 83 mg. of cod liver oil daily for vitamins A and D, non-saponifiable matter from 0.5 gm. of wheat germ oil weekly for vitamin E, and 50 mg. daily of Preparation 48-e (1) for vitamin F (the essential unsaturated fatty acids). The technique and materials used in this study were identical with those described (1) for the fat-free diet.

Gestation without Essential Unsaturated Fatty Acids—When hydrogenated coconut oil (free from linoleic acid) served as the source of energy instead of sucrose, the results were markedly simi-

---

* Aided by grants from the Research Board and the College of Agriculture of the University of California, and the Rockefeller Foundation.

[1] The coconut oil was furnished through the courtesy of Durkee Famous Foods, Inc., to whom we wish to extend our thanks.

[2] We wish to thank President R. M. Allen of the Vitamin Food Company, Inc., of New York for generously supplying a whole dried brewers' yeast remarkably high in its content of the antineuritic vitamin B.

TABLE I

Reproduction Data on Diet 675 Containing Hydrogenated Coconut Oil As Sole Source of Energy, with and without Essential Unsaturated Fatty Acids

Females between 5 and 8 months old were used; essential unsaturated fatty acids were fed when the rats were 6 to 7 months of age.

	Diet 675	Diet 675 + essential unsaturated fatty acids
Total No. of females in group	18	16
No. of females placed at least once with male	10	12
Total No. of instances of estrous female placed with male	26	23
No. of matings resulting	13	15
Per cent of matings	50.0	65.2
No. of matings showing sign of implantation	11	15
Per cent of matings showing sign of implantation	83.8	100.0
No. of instances with implantation sign; no litter resulted	2	0
Per cent of failures to litter after implantation sign	18.1	0
No. of litters	9	15
Per cent of matings resulting in litter	69.0	100.0
Average No. of young per litter	3.5	5.3
No. of young born dead	20	6
Per cent of young born dead	62.5	7.5
No. of young born living	12	74
Per cent of young born living	37.5	92.5
Average weight of young at birth, *gm.*	4.7	5.1
" " " mothers at time of littering, *gm.*	131	165
Per cent of animals having 22 day gestation period	11.1	86.0
Per cent of animals having 23 day gestation period	22.2	14.0
Per cent of animals having 24 day gestation period	33.3	0
Per cent of animals having 25 day gestation period	33.3	0
No. of mothers suckling young	0	10
Per cent of mothers suckling young	0	66.8

lar to those obtained on the fat-free diet, Diet 667 (1) (Table I). About 18 per cent of the pregnant females failed to litter after implantation. The characteristic prolonged gestation periods (1) were numerous, 22 per cent littering in 23 days, 33 per cent in 24 days, and 33 per cent in 25 days. The parturitions resulted in a large percentage of dead young, 62 per cent, and the living young were very weak, dying soon after birth. There was an average of 3.5 young per litter with the low average weight at birth of 4.7 gm. per animal. Suckling did not occur.

It is interesting that the outcome of gestations on this dietary regimen resembled strikingly that observed on the fat-free diet (1), although the mothers weighed on an average only 131 gm. in contrast to an average of 185 gm. for those on the fat-free diet. The superior body weight of rats having sucrose as their sole source of energy when compared with those receiving the saturated fatty acids as the sole source of energy has already been pointed out (3).

Gestation with Essential Unsaturated Fatty Acids—As in the case of the fat-free Diet 667, the addition of small amounts of the essential unsaturated fatty acids made a great difference in the course of gestation (Table I). There were no failures to litter after implantation, and no maternal mortality. Only 7.5 per cent of the young were born dead, and the average number per litter was increased from 3.5 to 5.3 and the average weight of the young increased from 4.7 to 5.1 gm. There were no very prolonged gestation periods, 86 per cent littering on the 22nd day and 14 per cent on the 23rd day. 67 per cent of the mothers nursed their young.

Lactation—Data on lactation were obtained on eight rats. No difference was noted with three rats on the usual supplements as contrasted with all the supplements increased in the case of two rats. Vitamins A, D, E, and F were trebled and an additional 0.8 cc. of Preparation 9-G and 0.5 cc. of autoclaved liver extract (1) were added to increase vitamins B and G respectively. In either case, the rats were weaned at 21 days with an average weight of approximately 25 gm. The loss in weight of the mothers was reduced, however, from an average of 16 gm. to 3 gm.

In the case of three rats, the mothers and young were transferred to our usual diet of natural foodstuffs (1) at littering. The young at weaning weighed 33 gm.; the mothers, instead of losing, gained an average of 49 gm. during the lactation period.

SUMMARY

1. Gestation is unsuccessful upon diets with saturated fatty acids (hydrogenated coconut oil) as the source of energy, when vitamin F (the essential unsaturated fatty acids) is absent.

2. The addition of vitamin F enables successful gestation to occur.

3. Lactation is not normal even when all the known supplements are increased.

BIBLIOGRAPHY

1. Evans, H. M., Lepkovsky, S., and Murphy, E. A., *J. Biol. Chem.*, **106**, 431 (1934).
2. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).
3. Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, **96**, 157 (1932).

VITAL NEED OF THE BODY FOR CERTAIN UNSATURATED FATTY ACIDS

VI. MALE STERILITY ON FAT-FREE DIETS*

By HERBERT M. EVANS, SAMUEL LEPKOVSKY, and ELIZABETH A. MURPHY

(*From the Institute of Experimental Biology, University of California, Berkeley*)

PLATE 1

(Received for publication, March 20, 1934)

INTRODUCTION

Burr and Burr (1) have shown that male rats on fat-free diets are unable to sire normal young; that, indeed, they do not usually copulate and that the occasional copulation that does take place is infertile. It seemed of importance to extend this work to determine the testicular histological changes in this type of dietary sterility and the possibility of reinvoking fertility.

EXPERIMENTAL

These experiments were conducted upon twenty-five males born from mothers which had been reared upon the fat-free Diet 667 (2) and had been given enough of the essential unsaturated fatty acids to enable successful gestation and lactation to occur. Two groups of these males (of eleven and of fourteen animals respectively) were reared without and with the essential unsaturated fatty acids. The essential unsaturated fatty acids were furnished by the daily administration of 50 mg. of a preparation made from corn oil (2) (Preparation 48-e). When the rats were between 5 and 6 months of age, every male of both groups was offered an estrous female on five successive nights and the next morning careful search was made for the presence of plug or spermatozoa in the vaginal canal of the female.

---

* Aided by grants from the Research Board and the College of Agriculture of the University of California, and the Rockefeller Foundation.

TABLE I

Sexual History of Sterile Males on Fat-Free Diet When Fed Essential Unsaturated Fatty Acids (Vitamin F) and Controls without Such Fatty Acids

Rat No.	Treatment	Weight of testes	Weight of liver	Weight of rat	Remarks
		gm.	*gm.*	*gm.*	
W-8467	No essential unsaturated fatty acids fed	0.77	6.01	132	No positive mating
W-8473	" "	0.79	5.41	110	" " "
B-8887	" "	0.95	6.78	165	" " "
W-9171	" "	0.92	6.38	192	" " "
GH-8604	" "	0.75	7.58	176	" " "
BH-9181	Received essential unsaturated fatty acids 16 wks.	2.27	7.82	232	Sired litters 15th and 16th wk. after receiving essential unsaturated fatty acids
BH-9182	" "	1.31	6.87	198	Plug, sperm, and r.b.c. 7th and 9th wk., litters 11th, 13th, and 16th wk. after receiving essential unsaturated fatty acids
W-8474	" "	1.00	7.92	204	No sperm found; plug found 7th, 10th, 13th, 15th, and 16th wk. after receiving essential unsaturated fatty acids
BH-8884	" "	1.28	5.34	182	Plug 9th wk., plug, sperm, and r.b.c. 11th wk., litters 13th and 15th wk. after receiving essential unsaturated fatty acids
Average of 14 rats	Reared with essential unsaturated fatty acids	2.63		249	9 sired young. 1 positive mating, r.b.c.; no litter. 4 negative matings; all had motile sperm in epididymis

Of the eleven rats reared without the essential unsaturated fatty acids, two were subjected to autopsy and the remaining nine rats on the unsupplemented fat-free diet were investigated to determine whether the sterility developing upon such purified diets could be cured by the administration of the essential unsaturated fatty acids. Four of the nine males were accordingly fed 50 mg. daily of Preparation 48-e. After they had received the curative supplement for 6 weeks each of the nine rats was offered an estrous female several times weekly every alternate week through an experimental period of 10 weeks, when they were all subjected to autopsy. The results of the experiment are given in Table I. Pertinent data obtained from rats reared with the essential unsaturated fatty acids are included for comparison.

Results

Of the fourteen males reared on the fat-free diet with the small daily supplement of the essential unsaturated fatty acids (50 mg.), ten copulated one or more times on five successive nights and nine of these ten sired twenty-one litters of young. The remaining four did not copulate, but on autopsy, motile spermatozoa were found in the epididymis of these animals as well as in all other members of this group (Table I). Histologically the testes of all these animals (Fig. 1) could not be distinguished from the testes of animals reared on natural foodstuffs. This confirmed strikingly the conception that only the small quantity of the essential unsaturated fatty acids which was fed is required for the preservation of normality of the reproductive system in males reared on these highly purified diets.

In the group of eleven males on the fat-free diet without the essential unsaturated fatty acids, the 55 attempted matings resulted in but a single copulation, as disclosed by a plug in which spermatozoa were absent. This evident impairment in sex interest is in contrast with its preservation for a significant portion of the life span in the male sterility occasioned by deficient vitamin E. This has already been pointed out by Burr and Burr (1) and is now confirmed by these studies. Autopsies were now performed on two males of the group; spermatozoa were not found in the epididymis, the reason therefor being evident when sections of the testes were prepared, for stages beyond the spermatocyte were not found in any of the tubules, all of which were in various stages of degeneration (Fig. 2). The majority of the tubules were lined with spermatogonia and one or more layers of spermatocytes. Multinucleated giant cells were frequent in the lumen of tubules and a considerable number of tubules devoid of epithelial investment were seen. But while the normal growth and transformation of the epithelium were thus prevented, forbidding especially the elaboration of ripe germ cells, the abundant residual epithelial tissue led us to expect restoration when the essential unsaturated fatty acids were added. This expectation was realized. Regeneration of testes as regards weight and function was strikingly produced by the administration of the essential unsaturated fatty acids.

Though sex interest was quickly restored in all the rats, only three out of the four rats became fertile. Matings were not attempted until the 7th week after the addition of the essential unsaturated fatty acids, by which time sex interest, but not yet fertility, was restored in two of the four cases (Rats BH-9182 and W-8474). In the two remaining cases, sex interest but not yet fertility was restored on the 9th week in one individual (Rat BH-8884), but in the other (Rat BH-9181), not until the 15th week, when fertility was also demonstrable. Three of the four rats became fertile, only one failing to sire young (Table I). Its testes were the lightest in weight but histological study showed it to possess many tubules with a completely restored epithelium (Fig. 3). The picture, therefore, resembles that seen in the much rarer cases of cured male sterility due to low vitamin E where many of the tubules are lined by a completely normal epithelium showing spermatogonia, spermatocytes, spermatids, and numerous spermatozoa, and other tubules are devoid of all cellular investment save the scattered scanty residual cells always seen after complete tubular degeneration in whatever way produced—elements usually designated as Sertoli's cells. Still other tubules showed one to three cell layers with or without the characteristic giant cells in their lumina. The three rats in which complete functional fertility had been proved possessed testes in which practically all the tubules, while small, had a completely normal epithelial investment, with here and there abundant ripe spermatozoa (Fig. 4), although in every case, in places, an occasional empty and badly shrunken tubule was encountered.

Our data have established the fact that grave degeneration of the testes occurs in the absence of the essential unsaturated fatty acids (Fig. 2), but that a regeneration of the epithelium of some tubules and a restoration of fertility can be provoked when the essential unsaturated fatty acids are fed after the testes have undergone extensive morphological impairment and proved sterility has supervened.

Thus in its amenability to a remedial diet, impairment of the male reproductive function due to deficiency in vitamin F resembles the impairment due to the deficiency in vitamin A (3) and is in contrast with impairment from lack of vitamin E. We can now state that in the case of vitamins A and F, structural and functional restoration results from curative feeding of the required vitamin after the existence of sterility has been proved, while restoration is much more difficult or impossible to achieve after proved sterility supervenes from deficiency in vitamin E.

SUMMARY

Males on fat-free diets invariably become sterile. The addition of small amounts of the essential unsaturated fatty acids can cure as well as prevent this sterility.

BIBLIOGRAPHY

1. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, **86**, 587 (1930).
2. Evans, H. M., Lepkovsky, S., and Murphy, E. A., *J. Biol. Chem.*, **106**, 431 (1934).
3. Mason, K. E., *Am. J. Anat.*, **52**, 153 (1933).

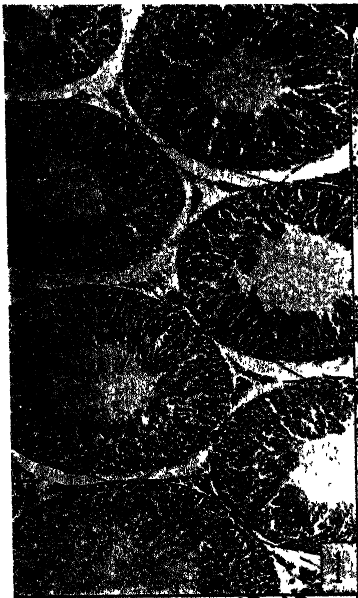
EXPLANATION OF PLATE 1

FIG. 1. Testis of Rat BH-9192 at 142 days of life when reared on fat-free diet plus the essential unsaturated fatty acids. × 94.

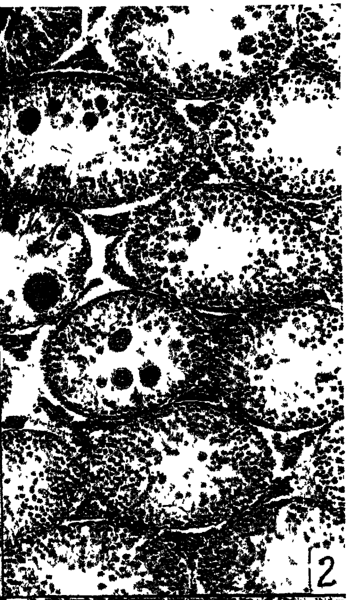
FIG. 2. Degeneration in testis of Rat W-9171 at 9 months of age reared on fat-free diet without the essential unsaturated fatty acids. × 94.

FIG. 3. Showing complete repair in some tubules and complete degeneration of others in testis of Rat W-8474 reared on fat-free diet for 9 months and receiving the essential unsaturated fatty acids for 16 weeks. × 94.

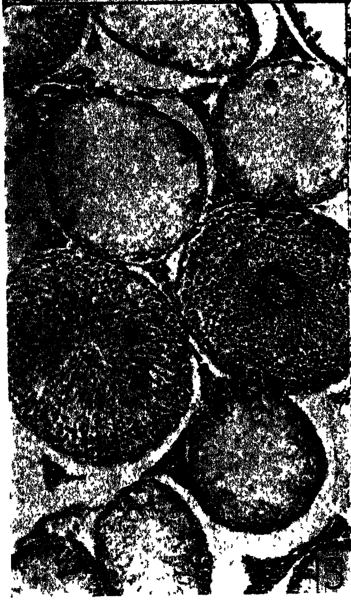
FIG. 4. Rat BH-8884 treated as was rat in Fig. 3, but showing practically complete repair of testis. (A few degenerative tubules were encountered.) × 94.



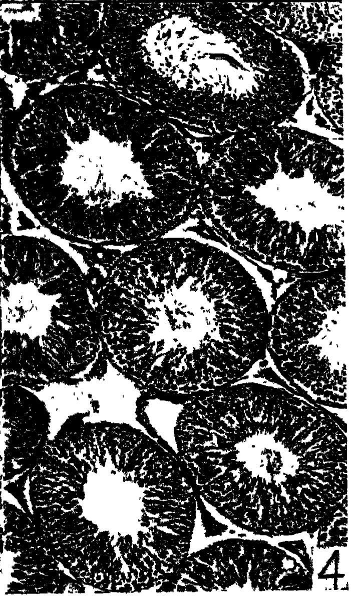
1



2



3



4

(Evans, Lepkovsky, and Murphy: Unsaturated fatty acids in diet. VI)

A MODIFICATION OF THE METHOD FOR DETERMINING METHIONINE IN PROTEINS

BY HARRY D. BAERNSTEIN

(From the Department of Physiological Chemistry, University of Wisconsin School of Medicine, Madison)

(Received for publication, May 25, 1934)

In 1932 (1) the author published a method for determining methionine in proteins based on the recovery of methyl iodide. Alcoholic silver nitrate was used as absorbant and the excess of silver nitrate was titrated with thiocyanate after filtering off the silver iodide. This method was quite satisfactory and has been used recently by Barritt (2) for the determination of methionine in wool. Certain difficulties in the handling of the alcoholic silver nitrate and in the treatment of the silver iodide were experienced, however, and therefore a search was made for a better absorbant.

My attention was called to the new method of Vieböck and Schwappach as modified by Clark (3), in which a mixture of glacial acetic acid, potassium acetate, and bromine is used to absorb methyl iodide in the determination of methoxyl groups. Methyl iodide is converted to iodate and the iodine liberated therefrom by potassium iodide and sulfuric acid is titrated with thiosulfate. This is considerably more accurate than the silver titration, and much simpler.

A complication arises, however, if the hydriodic acid used contains hypophosphite. During the heating the excess of hypophosphite is converted to phosphine, which is oxidized by the bromine to phosphate, and the scrubbers become clogged. In order to prevent this, a scrubber containing saturated mercuric chloride solution is introduced into the absorption train between the cadmium sulfate (or chloride) scrubber, and the acetic acid absorbers. In order to eliminate the small quantities of methyl iodide held back by these first two scrubbers, they are immersed in warm water kept heated by the stream which runs through the condenser.

Several minor changes have been made in the apparatus. The upper ground joint has been removed and all-glass absorbers have been substituted for those holding rubber stoppers.

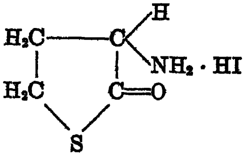
The recovery of methyl iodide from methionine is quantitative, the average for many determinations being 99.5 ± 2.0 per cent.

The rate of liberation of methyl iodide has been studied, and the results show that usually 3 hours are sufficient to continue the heating and aeration. The curve is practically identical for pure methionine and for proteins.

The question of the residue from methionine which remains behind in the acid is of some interest. On general principles one should expect an equivalent quantity of homocysteine. Butz and du Vigneaud (4), however, report only small amounts detectable with the Folin and Marenzi reagent (5). We have confirmed this. Homocystine is not a possibility, since, if formed, it would immediately be reduced by the hydriodic acid.

This finding is all the more surprising in view of the fact that the nitroprusside reaction of the digests for sulfhydryl is strongly positive and remains so indefinitely, although cysteine solutions similarly treated will lose their sulfhydryl reaction in a few hours. Furthermore, the digests of methionine after dilution will not reduce iodine, although cystine digests will.

In order to identify this methionine residue a preparation was made as follows: 1.0 gm. of methionine was boiled for 10 hours with 20.0 cc. of hydriodic acid (57 per cent containing 1.0 per cent H_3PO_2). The digest was evaporated to dryness on a water bath. The crystals which formed were dissolved in hot absolute alcohol and precipitated with 3 volumes of pure ether. A fine white powder was collected on the filter and washed with ether. This treatment suffices to give a pure product. On analysis this proved to be the thiolactone of homocysteine hydroiodide (Fig. 1).



Thiolactone hydroiodide

Calculated. —SH 13.47, NH_2-N 5.72, N 5.72, S 13.06, I 51.8
Found. " 13.17, " 5.72, " 5.47, " 13.01, " 50.4

This lactone, while more easily hydrolyzed than those of the oxygen series, requires more alkali than that contained in the Folin and Marenzi procedure.

The opening of the ring and the consequent liberation of the sulfhydryl group may be demonstrated with the nitroprusside reaction. If a little sodium nitroprusside is placed in a test-tube with about 2.0 cc. of a solution of thiolactone and 2 drops of concentrated ammonium hydroxide are added, no color will appear for about 15 seconds, after which it gradually deepens and reaches its full intensity in about 2 minutes. If the experiment be repeated with cysteine, the color appears at once upon the addition of ammonium hydroxide.

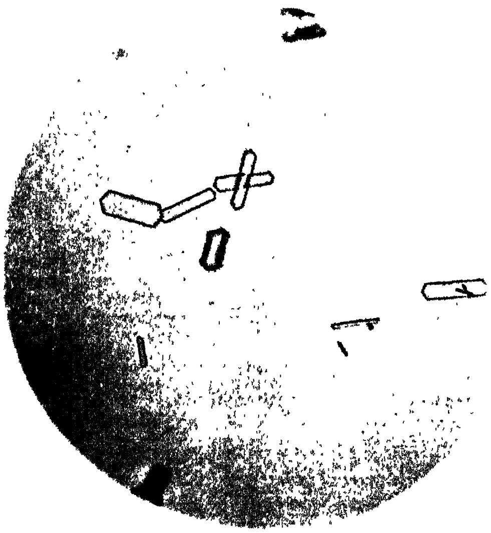


FIG. 1. Crystals of thiolactone of homocysteine hydroiodide

The ninhydrin reaction is interesting. In slightly acid solution thiolactone gives the usual blue color but cysteine hydrochloride gives a red color. In slightly alkaline solution thiolactone gives a red color as other amino acids do. We can therefore use this test only to prove the absence of the sulfhydryl group in thiolactone and the presence of α-amino nitrogen.

The Sullivan reaction (6) is negative on thiolactone, as was to be expected, since it has been shown that homocysteine is also negative. The reaction takes place in strong alkali in which thiolactone would be converted into homocysteine.

Recovery of Methyl Iodide and Thiolactone—Four flasks, each containing 20.0 mg. of methionine and 10.0 cc. of hydriodic acid (57 per cent containing 1.0 per cent hypophosphorous acid), were boiled on the reflux apparatus for 5 hours and aerated with CO_2. The condenser temperature was 50°. The methyl iodide was washed with 20 per cent cadmium chloride and a saturated solution of mercuric chloride and absorbed in 10.0 cc. of glacial acetic acid containing 10.0 per cent potassium acetate and 6 drops of bromine. A second such absorber is always used to insure complete recovery.

TABLE I

Determination of Methionine in Proteins

	Methionine	
	New method	Silver titration
	per cent	*per cent*
Ovalbumin	5.24	4.57
Edestin	2.39	2.07
Fibrin	2.59	2.40
Casein	3.50	3.53
Egg albumin (Merck)	5.10	5.29
Lactalbumin	2.62	2.63

At the end of the period the methyl iodide absorbers were rinsed into 100 cc. volumetric flasks containing 25.0 cc. of 25 per cent sodium acetate. A few drops of formic acid (sp. gr. 1.20) were added to reduce the excess bromine and the flasks whirled. 6 drops more of formic acid were added and the mixture was diluted to the mark. 25.0 cc. portions were added to 1.0 gm. of potassium iodide and 5.0 cc. of 10 per cent H_2SO_4 and the iodine was titrated with 0.05 N $Na_2S_2O_3$ with starch as indicator.

The digests were concentrated to about one-third their volume by simply draining the condensers while the flasks were boiling and connecting the outlet to a vertical condenser, kept cold. They were then rinsed into 50.0 cc. volumetric flasks with 0.5 N HCl which had been deaerated at the water pump.

5.0 cc. of the diluted digest were measured into 100 cc. volumetric flasks and 2.0 cc. of 20 per cent Na_2SO_3, 1.0 cc. of 25 per cent NaOH, and 5.0 cc. of the Folin and Marenzi cystine reagent were added. After standing 3 to 4 minutes the mixtures were diluted to the mark with 3 per cent Na_2SO_3 and compared with a cystine standard. This standard is made by diluting a stock solution of cystine in hydriodic acid with 0.5 N HCl.

The results of the iodine titration when calculated as methionine gave 19.95, 19.50, 20.4, and 19.8 mg., while the colorimetric method for thiolactone gave 19.5, 19.5, 19.8, and 18.8 mg.

Analysis of Proteins—0.5 gm. of protein is sufficient for a determination. The procedure is the same as given above with the analysis of the digest omitted, and the results obtained with several common proteins are given in Table I. These figures compare favorably with those previously reported.

It is possible to determine cysteine in the hydriodic acid digest. We are now working out the details and expect to publish the results soon.

By acidifying the cadmium chloride solution the hydrogen sulfide can be blown into standard iodine solution and thus this fraction can be also determined.

The origin of the hydrogen sulfide is still unknown. It might come from cystine, methionine, or sulfates, or some still unidentified constituent of proteins. The amounts obtained so far are very small.

The author wishes to thank Professor K. P. Link for recommending the Vieböck and Schwappach modification and Professor S. M. McElvain for suggesting the possibility of thiolactone formation.

SUMMARY

A new absorbant for methyl iodide is substituted for alcoholic silver nitrate in the determination of volatile iodide resulting from boiling proteins with hydriodic acid.

Mercuric chloride has been introduced in the absorption train to remove phosphine arising from hypophosphite used to keep hydriodic acid reduced.

A new derivative of methionine has been isolated from hydriodic acid digests of methionine in which the sulfhydryl group of homocysteine forms a thiolactone with the carboxyl group.

Several common proteins have been analyzed for methionine by the improved technique.

BIBLIOGRAPHY

1. Baernstein, H. D., *J. Biol. Chem.*, **97**, 663 (1932).
2. Barritt, J., *Biochem. J.*, **28**, 1 (1934).
3. Clark, E. P., *J. Assn. Off. Agric. Chem.*, **15**, 136 (1932).
4. Butz, L. W., and du Vigneaud, V., *J. Biol. Chem.*, **99**, 135 (1932–33).
5. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
6. Sullivan, M. X., and Hess, W. C., *Pub. Health Rep., U. S. P. H. S.*, suppl. 86 (1930).

THE DETERMINATION OF THE BASIC AMINO ACIDS IN SMALL QUANTITIES OF PROTEINS BY THE SILVER PRECIPITATION METHOD

By RICHARD J. BLOCK*

(*From the Department of Physiological Chemistry, Yale University, New Haven*)

(Received for publication, June 5, 1934)

The determination of arginine, histidine, and lysine among the products of protein hydrolysis was first successfully carried out by Kossel and Kutscher (1) in 1900. Their method has been repeatedly modified, especially by Vickery (2–7). In the present paper are described further modifications of this method, which permit relatively accurate determinations of the bases in quantities of proteins as small as 2 to 5 gm.

EXPERIMENTAL

Hydrolysis—2.50 gm. of protein are hydrolyzed with 25 cc. of dilute sulfuric acid (40 cc. of concentrated sulfuric acid added to 140 cc. of water) for 18 to 24 hours. The hydrolysate is cooled, transferred to a 250 cc. Pyrex centrifuge bottle, and diluted to about 100 cc. 30 gm. of $Ba(OH)_2 \cdot 8H_2O$ are dissolved in 100 cc. of hot water and poured into the hydrolysate. The resulting reaction of the solution should be between pH 3 and 6; if not, it must be adjusted to this range. The barium sulfate is centrifuged off and then suspended in 200 cc. of boiling water with the aid of a mechanical stirrer, the rapid stirring being maintained for 5 minutes. The suspension is again centrifuged and the precipitate is discarded. The filtrates are concentrated *in vacuo* at low temperature (50°) to 25 cc. The rate of distillation can be increased by the use of caprylic alcohol. All distillations, with one exception, are carried out in a special apparatus that permits rapid distillation in a vacuum at low temperature.

---

* Standard Brands, Inc., Fellow, 1932–34.

Histidine—The concentrated amino acid solution is transferred to a 100 cc. centrifuge tube. The filtrate and washings at this point should not exceed 50 cc. Saturated silver nitrate solution is added until a drop of the supernatant liquid gives a voluminous brown precipitate when added to a drop of barium hydroxide. Cold saturated barium hydroxide solution is slowly added with rapid stirring by an electric motor, until the reaction has reached pH 7.0 to 7.4. This is determined by the greenish blue color produced when a drop of brom-thymol blue solution is added. The stirrer must be stopped for this test. If the solution gives the faintest trace of a pink color with phenolphthalein, sulfuric acid must be added to pH 4 to 5 and the barium hydroxide precipitation repeated. The histidine silver precipitate is centrifuged off, suspended in water, and again centrifuged. The combined filtrates are immediately acidified with sulfuric acid (25 per cent) to pH 5 and concentrated *in vacuo* to 25 cc. (see "Arginine").

While these filtrates are being concentrated, the histidine silver precipitate is suspended in water, acidified to pH 5 to 6 (red to litmus paper), and decomposed by passing a stream of hydrogen sulfide into the bottom of the centrifuge tube while the suspension is being rapidly stirred. The reaction is complete as soon as the silver sulfide appears to coalesce, that is in 5 to 10 minutes. The precipitate is centrifuged, washed once with water, and the combined filtrates are concentrated *in vacuo* to 5 cc., poured into a 50 cc. centrifuge tube, and diluted to 10 cc. 30 cc. of a saturated solution of mercuric sulfate in 5 per cent (by weight) sulfuric acid are added to the warm solution (35–40°) and the tube is placed in the ice box for 3 to 5 hours. The histidine mercury percipitate is then centrifuged off and washed once with the mercuric sulfate reagent. The filtrates are discarded. The precipitate is suspended in water and decomposed by hydrogen sulfide as already described. In this case also, the end-point of the reaction is easily ascertained by the deep black color of the suspension and its speedy settling when the motor is stopped. The mercuric sulfide is centrifuged and washed, and the filtrates are concentrated *in vacuo* to 10 cc., neutralized to litmus paper with barium hydroxide, a pinch of barium carbonate is added, and the solution is heated to boiling under a reflux condenser. As soon as the solution has begun to boil, a slight excess of solid cupric hydroxide or carbonate is added, and the heating is continued 15 to 30 minutes longer. The supernatant liquid should be deep blue at this point. The histidine copper solution is cooled in ice water for ½ hour, after which the precipitate is centrifuged off and washed. Sufficient dilute sulfuric acid is added to the filtrate to cause the color to change to a yellow-green. The copper is removed by hydrogen sulfide and the purified histidine solution is concentrated *in vacuo* to 50 cc. The histidine can then be estimated by a nitrogen determination, by the Kapeller-Adler method (8)[1] or preferably by the isolation of the diflavianate. The isolation is accomplished after the remaining histidine solution is concentrated to 1 to 2 cc. *in vacuo* by the addition of 15 mg. of solid flavianic acid (2, 4-dinitro-1-naphthol-7-sulfuric acid) for every mg. of nitrogen present to the hot solution. The histidine diflavianate is allowed to crystallize in the ice box for 2 to 3 days. It is then filtered, washed with a little cold alcohol and with alcohol-ether mixture, and is finally dried at 100°. The histidine diflavianate must remain a pale yellow color after being dried; if any orange color develops, monoflavianate (9) is present and the determination is worthless. Should this occur the preparation is redissolved in the mother liquor and more flavianic acid is added.

Arginine—The concentrated amino acid solution remaining after precipitation of the histidine is transferred to a 100 cc. centrifuge tube and more silver nitrate is added if necessary. The arginine is then precipitated by the addition of hot saturated barium hydroxide to pH 13 to 14. It is very important to make this solution strongly alkaline. The arginine silver precipitate is centrifuged and washed once with cold saturated barium hydroxide. Lysine is isolated from the filtrate. The arginine silver precipitate is suspended in water, acidified to pH 5 with dilute sulfuric acid, and decomposed by hydrogen sulfide. The precipitate is centrifuged and washed once with water. The filtrates are concentrated *in vacuo* to 20 cc., poured into a 100 cc. centrifuge tube, and the reaction of the solution is readjusted to pH 5 to 6 with barium hydroxide. The precipitate is centrifuged, washed, and diluted to 100 cc. An aliquot part of this solution is removed for the determination of nitrogen. The remainder is concentrated *in vacuo* to 10 cc., heated to 90–100°, and the calculated amount of flavianic acid, dissolved in the minimal amount of hot water, is added (6 mg. of flavianic acid are used for every mg. of nitrogen in the arginine fraction). The solution is cooled, placed in the ice box for 2 to 3 hours, and the arginine flavianate is filtered, washed with ice water, alcohol, and ether, dried at 100° for a few minutes, and weighed.

---

[1] I am indebted to Dr. F. A. Csonka for suggesting the use of this method. He had successfully employed it for some time before my previous publication (13).

Lysine—The strongly alkaline filtrate from the arginine silver precipitation is immediately acidified to pH 1 with sulfuric acid and the silver is removed by hydrogen sulfide. The precipitate is washed, and the combined filtrates are poured into a 1 liter round bottom flask. The amino acid solution is concentrated to 5 to 10 cc., a few drops of a 1 per cent alcoholic solution of phenolphthalein are added, together with sufficient 40 per cent sodium hydroxide to produce a reddish purple color; 25 to 50 cc. of alcohol are added and the ammonia is removed by concentration *in vacuo*. The solution is concentrated to 5 cc., and sufficient 20 per cent sulfuric acid is added to discharge the red color. The acid solution is poured into a 100 cc. centrifuge tube, diluted to 29 to 30 cc., and 1.1 cc. of concentrated sulfuric acid are added; any small precipitate of silver sulfide or barium sulfate is centrifuged off and washed with 1 to 2 cc. of water. The clear solution is then transferred to another 100 cc. centrifuge tube, heated on the steam bath to 90–95°, and 50 cc. of a hot 20 per cent solution of phospho-24-tungstic acid (Mallinckrodt) in 5 per cent (by weight) sulfuric acid are added with stirring. No precipitate should form during the addition of the phosphotungstic acid. The solution is quickly cooled and placed in an ice bath for 30 minutes. The crystalline lysine phosphotungstate is centrifuged, suspended in a 2 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid by means of the electric stirrer, and again centrifuged. This washing is repeated once more. The lysine phosphotungstate is suspended in distilled water containing 1.0 cc. of sulfuric acid and is decomposed by extracting the phosphotungstic acid by means of an amyl alcohol-ether mixture (1250 cc. of ether, 1000 cc. of amyl alcohol, 50 cc. of ethyl alcohol). The aqueous phase is extracted three times by the alcohol mixture, the combined organic solvents are reextracted with water containing a drop of sulfuric acid, and this water layer is again extracted with amyl alcohol-ether. The combined aqueous solutions are finally extracted with a fresh portion of organic solvent. The clear, colorless lysine sulfate solution is washed into a 250 cc. centrifuge tube and 5 gm. of $Ba(OH)_2 \cdot 8H_2O$ dissolved in 20 cc. of hot water are added. The solution must be acid to litmus at this point. The lysine solution is warmed on the steam bath for a few minutes and 2.0 gm. of powdered barium carbonate are added. After the carbonate is stirred for a few seconds, the precipitate is centrifuged and washed once with water. The combined filtrates are transferred to a 1 liter flask, together with 3 cc. of caprylic alcohol and 1.0 gm. of barium carbonate. The solution is concentrated *in vacuo* to 50 cc., the precipitate is centrifuged and washed with water, and the solution is diluted to 100 cc. The nitrogen content is determined on a small aliquot part. The rest of the lysine carbonate solution is concentrated *in vacuo* in a Claisen flask with a detachable neck (Scientific Glass Apparatus Company, No. 1390) to 1 to 2 cc. The capillary and sides of the vessel are washed down with 5 to 10 cc. of 93 per cent alcohol. If more than a slight amount of a white flocculent precipitate appears, a drop or two of water should be added. The lysine solution is

TABLE I

Basic Amino Acids of Proteins

Protein	Investigator	Amount hydrolyzed	Histidine	Arginine	Lysine
		gm.	*per cent*	*per cent*	*per cent*
Horse hemoglobin	Author (13)	3.37	7.5	3.2	8.1
" "	Vickery and Leavenworth (14)	199.50	7.6	3.3	8.1
Edestin....................	Author	4.00	2.1	15.6	2.1
"	Vickery and Leavenworth (5)	366.3	2.08	15.8	2.2
Serum Albumin A...........	Author	4.60	1.90	4.6	9.1
" " B...........	"	4.60	1.92	4.8	9.0

cooled to room temperature and 8.1 mg. of purified picric acid (10) dissolved in warm alcohol are added for every mg. of lysine nitrogen. The lysine picrate, which should crystallize out immediately on cooling, is placed in the ice box for 2 to 3 hours, filtered, washed with a cold alcohol-ether (1:1) solution, and dried at 100° for a few minutes, and weighed.

The results of the analysis may be calculated from the following factors: histidine diflavianate × 0.198 = histidine; arginine flavianate × 0.3566 = arginine; lysine picrate × 0.39 = lysine.

A complete analysis may be conducted in 8 working hours after some experience has been obtained.

As an illustration of the accuracy and reproducibility of the results, the data in Table I are given. Many analyses carried out by this technique have been published elsewhere (10–13).

BIBLIOGRAPHY

1. Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, **31**, 165 (1900).
2. Kossel, A., and Patten, A. J., *Z. physiol. Chem.*, **38**, 39 (1903).
3. Kossel, A., and Gross, R. E., *Z. physiol. Chem.*, **135**, 167 (1924).
4. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **72**, 403 (1927); **83**, 523 (1929).
5. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **76**, 707 (1928).
6. Vickery, H. B., and Block, R. J., *J. Biol. Chem.*, **86**, 107 (1930); **93**, 105 (1931).
7. Calvery, H. O., *J. Biol. Chem.*, **83**, 631 (1929).
8. Kapeller-Adler, R., *Biochem. Z.*, **264**, 131 (1933).
9. Vickery, H. B., *J. Biol. Chem.*, **71**, 303 (1926–27).
10. Block, R. J., *J. Biol. Chem.*, **104**, 339 (1934).
11. Block, R. J., *J. Biol. Chem.*, **104**, 343 (1934); **105**, 455 (1934).
12. Block, R. J., Darrow, D. C., and Cary, M. K., *J. Biol. Chem.*, **104**, 347 (1934).
13. Block, R. J., *J. Biol. Chem.*, **105**, 663 (1934).
14. Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.* **79**, 377 (1928).

STUDIES OF ACIDOSIS

XXII. APPLICATION OF THE HENDERSON-HASSELBALCH EQUATION TO HUMAN URINE*

BY JULIUS SENDROY, JR., S. SEELIG,† AND DONALD D. VAN SLYKE

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, June 15, 1934)

The Henderson (1909–10) equation

$$[\mathrm{H}^+] = K' \frac{[\mathrm{Ha}]}{[\mathrm{Ba}]} \tag{1}$$

relating hydrion concentration in buffer acid solutions to the ratio, free acid:alkali salt, has been frequently applied to the calculation of pH from H_2CO_3 and $BHCO_3$ in the blood, particularly since Hasselbalch (1916) transformed the equation for this purpose into logarithmic forms.

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{[\mathrm{BHCO_3}]}{[\mathrm{H_2CO_3}]} \tag{2}$$

or

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{[\mathrm{CO_2}] - 0.0591\alpha p}{0.0591\alpha p} \tag{3}$$

$[BHCO_3]$, $[H_2CO_3]$, and $[CO_2]$ indicate millimolar concentrations of bicarbonate, free carbonic acid, and total CO_2 respectively; α is the Bunsen solubility coefficient of CO_2; p is the CO_2 tension in mm. of mercury; $0.0591\ \alpha p = [H_2CO_3]$; pK′ is the negative logarithm of the apparent first dissociation constant of carbonic acid.

---

* A preliminary communication concerning this work was delivered at the XIVth International Physiological Congress at Rome (Sendroy, Seelig, and Van Slyke, 1932).

† Fellow of the Rockefeller Foundation on leave of absence from the Charité Krankenhaus, Berlin, Germany.

The accuracy with which the equation can be applied depends upon the exactness with which the constants, pK′ and α, are known for the particular solution investigated. For serum a number of authors have determined the value of pK′, and the results have been summarized by Hastings, Sendroy, and Van Slyke (1928), who found the value to be 6.10 ± 0.03. The value of α for normal serum has also been determined and found to be quite constant at 0.510, 93.4 per cent of the value in water (Van Slyke, Sendroy, Hastings, and Neill, 1928).

For studies of urine, on the other hand, there has been relatively little application of this equation. Gamble (1922), who used Equation 2 to calculate H_2CO_3 content from urine pH and CO_2 content, appears to be the first author to apply it to a physiological investigation. He used the same value for pK′, 6.1, which had been found for serum. He did not calculate CO_2 tension values, and hence did not require a value for α. Mainzer and Bruhn (1931) later determined α and pK′ values in twenty urine specimens, and found such wide variations in these values that accurate application of the Henderson-Hasselbalch equation to urine by the use of average α and pK′ values appeared to be impossible. Mainzer and Bruhn, for their pK′ calculations, determined pH with the quinhydrone electrode. Their α values were determined by a technique modeled in most details after that used by Van Slyke, Sendroy, Hastings, and Neill (1928). Mainzer and Bruhn's values of α and pK′ have thus far remained the only ones in the literature.

In the present paper we have redetermined the values of α and pK′ for human urine, with precautions outlined below. The pK′ values have been determined with the standard hydrogen electrode.

EXPERIMENTAL

The Bunsen solubility coefficient, α, was obtained by practically the same procedure used by Van Slyke, Sendroy, Hastings, and Neill (1928), in estimating it for serum, except that in most of the present determinations an analyzed mixture of CO_2 and air was used as the saturating gas instead of pure CO_2. The same results were obtained by both procedures. The CO_2 content of the air-CO_2 mixtures, at the end of saturation, was measured by analysis, either in the Haldane apparatus or in the manometric apparatus according to Van Slyke, Sendroy, and Liu (1932). Before saturation, the urines were acidified with HCl to a pH below 3.5. The applicability of HCl, and its relatively slight effect on α, have been discussed on p. 783 of Van Slyke, Sendroy, Hastings, and Neill (1928). Values of α were calculated according to Equation 4 (Equation 2 of the latter authors).

$$(4) \qquad \alpha = \frac{[CO_2]}{100} \times \frac{760}{p} \times \frac{V_R}{V_{38^\circ}} = \frac{[CO_2]}{p} \times 7.57$$

V_R and V_{38° represent respectively the volumes occupied by 1 gram of the solution at room temperature, when analyzed, and at 38°, the temperature of saturation. The factor 7.57 includes the value of V_R/V_{38° when the room temperature is 24–27°. Solubility coefficients calculated by Equation 4 were subsequently corrected for the slight effect of the added HCl.

The value of pK′ was obtained essentially by the procedure applied by Hastings, Sendroy, and Van Slyke (1928) for determination of pK′ in serum. For each experiment, enough urine for several analyses and fillings of the electrode vessel was saturated at 38° with a mixture of CO_2 and H_2. After saturation was complete, a portion of the gas mixture was analyzed for CO_2. The rest of the same gas mixture was then used in the pH determinations made in the Clark-Cullen electrode vessel. Duplicate pH values uniformly agreed within 0.005 unit. The CO_2 contents of the saturated urines were determined with the Van Slyke-Neill apparatus as described on p. 299 of Peters and Van Slyke (1932). Samples of appropriate size were taken to secure the maximum accuracy for values of $[CO_2]$ of 1 part in 300.

Total base was determined in the urine by a modification of Fiske's (1922) method. The phosphates were removed by Hoffman's procedure (1931), the bases were ashed to sulfates, and the SO_4 was weighed as $BaSO_4$. Enough urine, 10 or 15 cc., was used to yield 0.2 or 0.3 gram of $BaSO_4$.

Specific gravity readings were made with an ordinary bulb hydrometer. The gravities were read to 0.001, and are thus recorded, although calibration of the instrument against determinations by weight indicated its accuracy to be limited to 0.003.

RESULTS

Solubility of CO_2 in Urine at 38°

Our twenty-two values for α in Table I vary from 0.536 to 0.498, with an average of 0.522 ± 0.008. Figs. 1 and 2 show the decrease in α with increasing total base content and specific gravity. The dashed lines in Fig. 1 indicate the solubilities which Van Slyke, Sendroy, Hastings, and Neill (1928) found in aqueous chloride

TABLE I

Values of CO_2 Solubility Coefficient in Human Urine at 38°

Urine No.	Total base	Specific gravity	CO_2 tension at 38°	CO_2 content at 24-27°	α*
	m.-eq. per l.		*mm.*	*vol. per cent*	*cc. CO_2 per cc. solution*
1	255	1.030	91.1	6.28	0.524
2	246	1.024	122.3	8.08	0.504
3	63	1.008	100.7	7.11	0.536
4	247	1.031	109.6	7.24	0.503
5	254	1.035	710.8	46.48	0.498
6	193	1.018	110.6	7.60	0.524
7	68	1.011	109.2	7.56	0.528
8	76	1.012	107.0	7.34	0.523
9	140	1.023	109.5	7.62	0.531
10	222	1.026	111.2	7.51	0.515
11	93	1.009	110.8	7.74	0.532
12	151	1.013	111.3	7.59	0.520
13	79	1.007	108.6	7.57	0.530
14	54	1.019	107.0	7.43	0.529
15	157	1.010	105.2	7.27	0.527
16	176	1.019	110.1	7.50	0.518
17	109	1.025	109.9	7.64	0.530
18	136	1.019	107.0	7.34	0.522
19	125	1.015	108.9	7.49	0.524
20	95	1.020	108.9	7.56	0.530
21		1.017	107.6	7.24	0.513
22	60	1.010	105.7	7.39	0.532
Average..					0.522

* Corrected for effect of added HCl.

and acid phosphate solutions. Most of the urine values fall between those for solutions of KCl and NaH_2PO_4 of the same total base concentration. Urea, the only non-electrolyte normally present in sufficient concentration to affect α significantly, in maximal concentration (0.5 M or 3 per cent) lowers α by only 0.008 unit (Usher, 1910). Hence it is to be expected that the lowering of α of urine below that of water is chiefly a function of the salts, of which ordinarily the most abundant are chloride and acid phosphate.

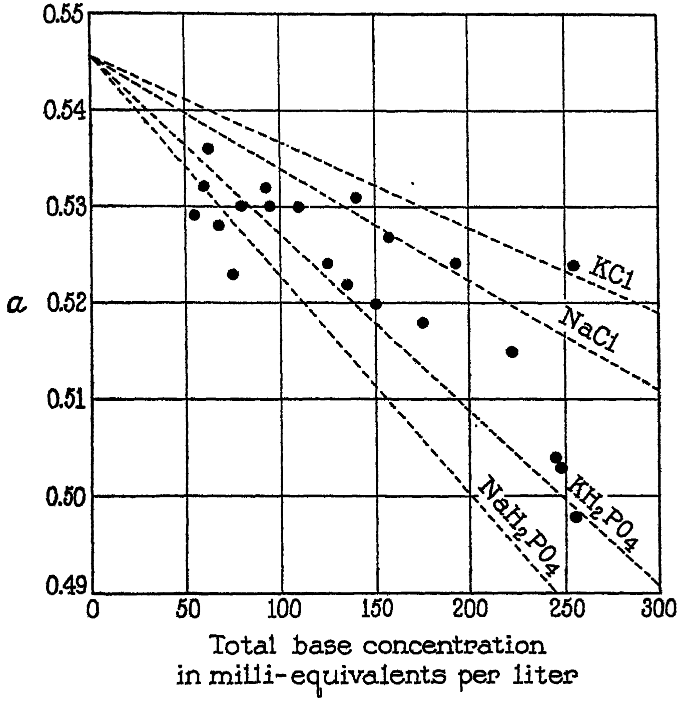


FIG. 1. The relationship between the CO_2 solubility coefficient of urine and its salt content. All points are for urine. Dotted lines are for salts, taken from Van Slyke, Sendroy, Hastings, and Neill (1928).

Mainzer and Bruhn's (1931) values of α for twenty urines extend from 0.441 to 0.514, with an average of 0.482. Their average is 8 per cent below ours, and a number of their values are lower than any we obtained in the most concentrated urines.

Mainzer and Bruhn's technique, like ours, followed in general that of Van Slyke, Sendroy, Hastings, and Neill (1928); but Mainzer and Bruhn introduced the following changes first. They kept their samples under mineral oil saturated with the equilibrating gas mixture of CO_2 and air, instead of confining them in closed containers over mercury. Second, in transferring their samples of urine to the manometric apparatus, they apparently used open pipettes instead of the special closed pipette described in Section XII of Van Slyke and Neill (1924). It is possible that these differences in technique may have caused CO_2 losses and the comparatively low results of Mainzer and Bruhn.

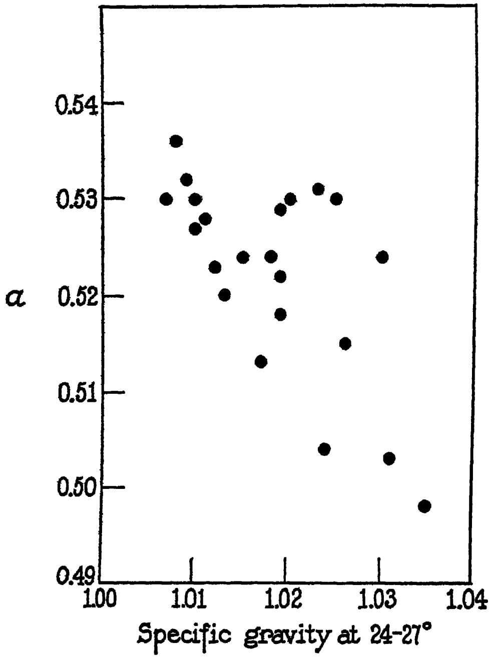


FIG. 2. The relationship between the CO_2 solubility coefficient of urine and its specific gravity.

pK′ of Urine at 38°

Our data in Table II give pK′ values in urine averaging 6.10 ± 0.07. The maximum deviation from the average value is ±0.13.

TABLE II

*Values of pK' in Human Urine at 38°**

Urine No.	CO_2 content analyzed (a)	CO_2 tension analyzed (b)	$[H_2CO_3]$ from individual α and (b) (c)	$[BHCO_3]$ from (a) − (c) (d)	*E* corrected to 760 mm. H_2 pressure (e)	E_0 (f)	pH determined (g)	pK' (h)
	mM per l.	*mm.*	*mM per l.*	*mM per l.*	*millivolts*	*millivolts*		
1	2.030	59.6	1.845	0.185	558.2	236.6	5.213	6.212
2	69.38	59.1	1.760	67.62	707.5	236.6	7.633	6.048
3	3.813	59.7	1.891	1.922	616.2	236.8	6.150	6.143
4	2.291	62.0	1.843	0.448	568.7	236.8	5.480	5.995
5	2.216	58.3	1.715	0.501	576.5	237.1	5.502	6.036
6	13.40	59.5	1.843	11.56	660.5	236.8	6.868	6.070
7	1.993	58.2	1.816	0.177	554.1	237.1	5.139	6.150
8	1.970	58.1	1.792	0.178	551.8	236.7	5.108	6.110
9	13.33	59.9	1.880	11.45	660.9	236.7	6.876	6.091
10	2.096	61.4	1.870	0.226	559.9	236.6	5.241	6.159
11	3.387	61.7	1.940	1.447	608.2	236.8	6.020	6.147
12	16.63	58.6	1.801	14.83	668.4	236.7	6.998	6.082
13	3.105	58.4	1.831	1.374	595.3	236.5	5.816	5.973
14	2.189	58.2	1.819	0.370	573.0	236.6	5.453	6.144
15	15.51	58.9	1.835	13.67	667.3	236.7	6.980	6.108
16	4.897	58.5	1.791	3.106	624.7	236.4	6.294	6.055
17	1.990	56.2	1.761	0.229	589.8	236.3	5.244	6.130
18	2.115	59.4	1.833	0.282	564.1	236.8	5.306	6.119
19	2.031	58.6	1.815	0.216	556.3	236.8	5.179	6.103
20	8.38	60.2	1.885	6.49	648.2	236.7	6.670	6.133
21	2.371	60.3	1.827	0.544	575.8	236.4	5.501	6.027
22	2.333	60.3	1.912	0.421	577.2	236.4	5.524	6.182
Average...								6.101

* Individual values of α and total base for each urine are given in Table I.

Fig. 3 shows that the variations of pK' in urine are related to variations in total base content. Hastings and Sendroy (1925) have shown that pK'_1 of carbonic acid, in NaCl-$NaHCO_3$ solutions containing mainly NaCl, can be expressed by the equation

$$pK' = 6.32 - 0.5\sqrt{\mu} \tag{5}$$

where μ is the ionic strength, 6.32 is the pK' (= pK) at infinite dilution of the salts,[1] and the term $0.5\sqrt{\mu}$ expresses the depression of pK′ caused by salts. With univalent salts, like NaCl and $NaHCO_3$, the ionic strength is the same as the total base concentration in equivalents per liter. With polyvalent salts, the ionic strength is greater, being three times as great in the case of Na_2SO_4, or of $CaCl_2$. Since the urine electrolytes are in part polyvalent, it could be anticipated that, per unit total base concentration, the pK′ would be decreased somewhat more than in a NaCl-$NaHCO_3$ solution. Fig. 3 shows in fact that the equivalent depression in urine is somewhat greater, and that the relationship of pK′ to total base concentration is expressed by the equation

---

[1] Correction by use of the later CO_2 factors of Van Slyke and Sendroy (1927) and the α values of Van Slyke, Sendroy, Hastings, and Neill (1928) affects the results of Hastings and Sendroy (1925) just enough to change their pK from 6.33 to 6.32.

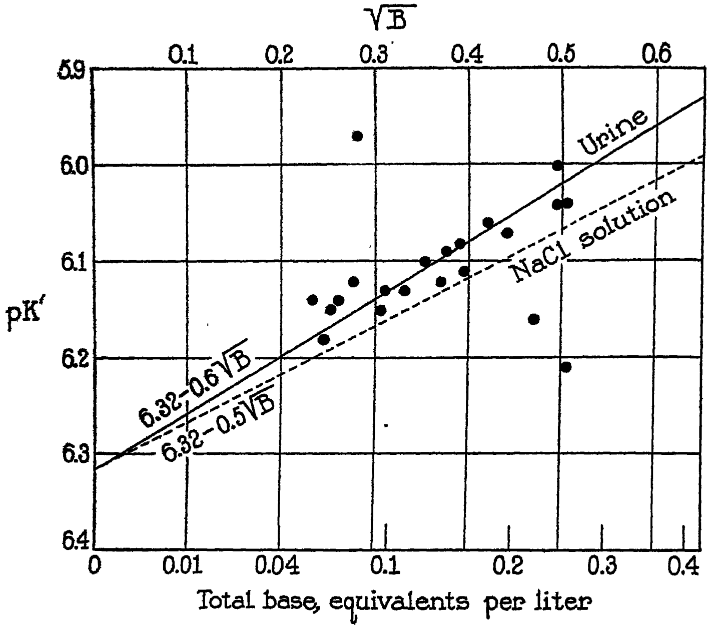


FIG. 3. The variation of urine pK′ with electrolyte (total base) concentration. All points and solid line are for urine. Dotted line for NaCl is taken from Hastings and Sendroy (1925).

$$pK' = 6.32 - 0.6\sqrt{B} \tag{6}$$

where B represents the total base content in equivalents per liter. In eighteen of the twenty-two determinations Equation 6 expresses the value of pK′ within ±0.03 unit of the observed value.

Mainzer and Bruhn (1931) obtained pK′ values varying from 5.81 to 6.30, and averaging 6.13. They were unable to find any correlation between pK′ variation and freezing point lowering. Their average is near ours, and it is possible that in extremely dilute urines pK′ may approach as close to the pK for infinite dilution as their maximum value of 6.30. It is difficult to conceive, however, factors which could lower pK′ to Mainzer and Bruhn's minimum value. Total base rarely exceeds 0.4 equivalent per liter, which would correspond, by Equation 6, to a pK′ of 5.94. The technical error in determining pK′ is likely to be much greater in urines than in serum, or solutions of bicarbonate content similar to that of serum. The $BHCO_3$ in the urine is often only a fraction of a millimole per liter, and may be as little as 0.1 of the H_2CO_3. Consequently slight errors in calculating the H_2CO_3 from the CO_2 partial tension and the α used for the individual urine, would be multiplied in some cases as much as 10-fold in their effect on $BHCO_3$ calculated by difference. Of our own three pK′ values (Urines 1, 10, 13) which deviate markedly from Equation 6, two are from experiments in which the $BHCO_3$ concentration was only 0.3 mM or less. Thus, the deviations may in part be the result of the wider margin of experimental error that is unavoidable when $BHCO_3$ is so low. It appears possible that the wider fluctuations of Mainzer and Bruhn may have been caused by a somewhat wider margin of error in some of their determinations. A low value of α used in Equation 3 would cause a low value of pK′, and we have pointed out above that some of their α values appear to be improbably low.

Effect of Urea on pK′—Table III contains data indicating the effect which urea in 3 per cent (0.5 M) concentration has on the value of pK′ in bicarbonate-salt solution. The procedure followed for these experiments was exactly the same as that of Hastings and Sendroy (1925). The calculations were made according to Equations 1 and 2. The value of α used was calculated for each solution according to its composition, from the data of Van Slyke, Sendroy, Hastings, and Neill (1928) for the salt effect, and according to Usher (1910) for the urea effect. A urea concentration of 0.5 M was used because this approximates the maximum urea content of human urine. Urea in this concentration causes an average decrease in pK′ of only 0.013 unit. Variations in pK′ of urine

Table III

Effect of Urea on pK′ of Salt Solution at 38°

Sample No.	Initial composition				CO_2 tension	α	$[H_2CO_3]$	$[CO_2]$	$[NaHCO_3]$	pH	pK′	Average pK′	Δ pK′ per 500 mM urea per liter
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mm.*		*mM per l.*	*mM per l.*	*mM per l.*				
1	$NaHCO_3$, 28.6				97.5	0.542	3.123	31.97	28.85	7.194	6.228	6.219	
1-a	" 28.6				95.4	0.542	3.057	31.93	28.87	7.185	6.209		
2	" 28.6	Urea, 500			97.6	0.534	3.083	31.83	28.75	7.176	6.206	6.216	
2-a	" 28.6	" 500			96.7	0.534	3.092	31.90	28.81	7.196	6.226		−0.003
3	$NaHCO_3$, 28.6		NaCl, 128.0		96.6	0.528	3.016	31.85	28.83	7.099	6.119		
4	" 28.6	Urea, 500	" 128.0		96.1	0.520	2.960	31.65	28.69	7.099	6.112	6.100	
4-a	" 28.6	" 500	" 128.0		97.2	0.520	2.989	31.82	28.83	7.073	6.088		−0.019
5	Na_2CO_3, 15.0		Na_2HPO_4, 24.0	KH_2PO_4, 6.0	48.7	0.534	1.538	30.06	28.52	7.428	6.159		
6	" 15.0	Urea, 500	" 24.0	" 6.0	30.4	0.526	0.946	27.90	26.95	7.596	6.142		−0.017
7	$NaHCO_3$, 30.0		Na_2HPO_4, 21.6	KH_2PO_4, 8.4	87.6	0.534	2.765	33.48	30.71	7.196	6.150		
8	" 30.0	Urea, 500	" 21.6	" 8.4	85.7	0.526	2.665	33.45	30.78	7.202	6.139		−0.011
Average...													−0.013

exceeding 0.01 can therefore not be ascribed to variations in the urea concentration.

TABLE IV

Effect of Variation in α and pK′ on Accuracy of Henderson-Hasselbalch Equation Applied to Urine

Urine No.	CO$_2$ content	CO$_2$ tension	H$_2$CO$_3$ calculated		BHCO$_3$ calculated		pH calculated from (*a*) and (*b*) with		Error in pH calculated according to (*h*)
			From individual α (Table I) and (*b*)	From average α of 0.522, and (*b*)	From (*a*) − (*c*)	From (*a*) − (*d*)	Individual α and pK′ (Tables I and II)	Average α and pK′ of 0.522 and 6.10	(*g*) − (*h*)
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)
	mM per l.	*mm.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*			
1	1.928	56.8	1.758	1.752	0.170	0.176	5.20	5.10	−0.10
2	75.37	119.0	3.542	3.671	71.83	71.70	7.36	7.39	+0.03
3	3.360	41.6	1.318	1.283	2.042	2.077	6.33	6.31	−0.02
4	2.115	59.4	1.764	1.832	0.351	0.283	5.29	5.29	0.00
5	2.481	68.8	2.023	2.122	0.458	0.359	5.39	5.33	−0.06
6	16.90	124.3	3.848	3.835	13.05	13.06	6.60	6.63	+0.03
7	1.898	52.6	1.641	1.623	0.257	0.275	5.35	5.33	−0.02
8	1.441	41.5	1.282	1.280	0.159	0.161	5.20	5.20	0.00
9	18.20	124.5	3.906	3.841	14.29	14.36	6.65	6.67	+0.02
10	2.595	69.8	2.123	2.153	0.472	0.442	5.51	5.41	−0.10
11	3.016	54.6	1.716	1.684	1.300	1.332	6.04	6.00	−0.04
12	18.28	73.0	2.242	2.252	16.04	16.03	6.94	6.95	+0.01
13	2.810	51.1	1.599	1.576	1.211	1.234	6.09	5.99	−0.10
14	2.127	56.9	1.778	1.755	0.349	0.372	5.43	5.43	0.00
15	16.22	76.8	2.391	2.369	13.83	13.85	6.87	6.87	0.00
16	6.009	84.0	2.571	2.591	3.438	3.418	6.18	6.22	+0.04
17	2.110	51.0	1.597	1.573	0.513	0.537	5.64	5.63	−0.01
18	2.621	74.6	2.301	2.301	0.320	0.320	5.26	5.24	−0.02
20	9.75	86.2	2.698	2.659	7.05	7.09	6.54	6.53	−0.01
21	2.724	69.6	2.108	2.147	0.616	0.577	5.49	5.53	+0.04
22	2.188	59.1	1.857	1.823	0.331	0.365	5.43	5.40	−0.03
Average									±0.032

Accuracy of Henderson-Hasselbalch Equation Applied to Urine

Table IV indicates the accuracy with which the *average* α and pK′ values may be applied to urine acid-base balance calculations by Equation 3. The pH values in column (*h*) have been calculated by the use of average α and pK′; those in column (*g*) have been derived by the use of the individually determined α and pK′ values of the same urines, as given in Tables I and II. Evidently urine pH may be calculated from accurately determined CO_2 content and CO_2 tension values, with an exactness at least equalling that of the colorimetric method (Hastings, Sendroy, and Robson, 1925).

Construction of Nomogram of Fig. 4

For calculation of one of the three variables, CO_2 content, CO_2 tension, or pH from the other two in urine, a line chart for graphic solution of the Henderson-Hasselbalch equation is convenient. The nomogram in Fig. 4 accurately presents total CO_2 as a linear function of pH and CO_2 tension. It is theoretically exact, and is in this respect an improvement over the blood plasma line chart of Van Slyke and Sendroy (1928, p. 783). A straight line drawn through given points on two of the scales will intercept the third scale at a point corresponding to the relationship expressed by Equation 3, with $\alpha = 0.522$ and $pK' = 6.10$. By the use of appropriate α and pK′ values, a similar chart could be constructed for blood plasma calculations.

If the fraction of the total CO_2 in the form of the free acid, symbolized for convenience as H_2CO_3, be denoted by f, we may write

$$(7) \qquad f = [H_2CO_3]/[CO_2], \text{ and } \log f = \log [H_2CO_3] - \log [CO_2]$$

We have then, a straight line logarithmic relationship in the form of $\log x = \log y + \log z$. If Equation 2 be written in the form

$$(8) \qquad pH = pK' + \log \frac{1-f}{f}$$

then it is obvious that we have another logarithmic expression for f; namely,

$$(9) \qquad \log f = pK' - pH + \log (1-f)$$

Since given values of $[CO_2]$ and $[H_2CO_3]$ (or CO_2 tension) fix the value of f they also fix the value of pH.

For Fig. 4, the values of $\log [CO_2]$ and $\log [H_2CO_3]$ in terms of millimoles per liter, were spaced off on parallel axes of equal length

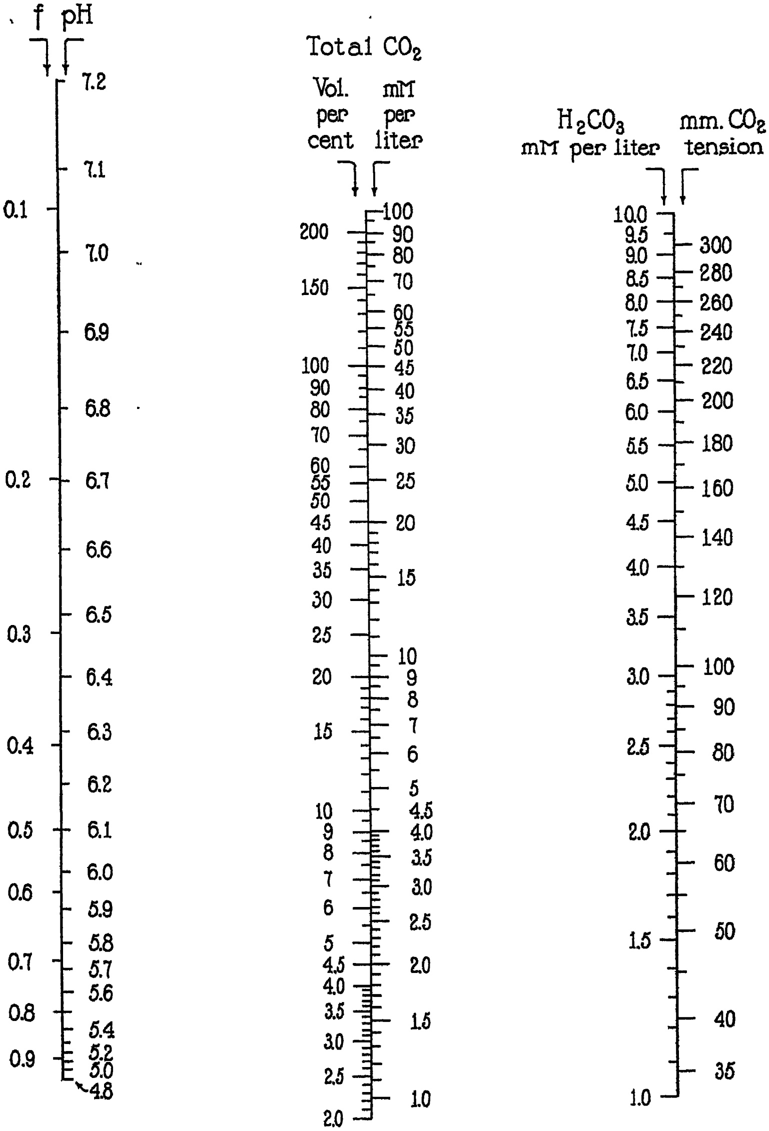


Fig. 4. Nomogram for calculation of acid-base balance of urine. A straight line drawn between values on any two scales intersects the third at a value corresponding to the relationship expressed by the Henderson-Hasselbalch equation.

drawn on rectangular coordinate paper. An equal distance away, another parallel axis of equal length was spaced off for values of log f. By Equation 8 values of f corresponding to given pH values were calculated, and located logarithmically on the f axis. The points found were marked with values of the given corresponding pH. Points corresponding to CO_2 tension values were marked off on the H_2CO_3 scale. For these calculations the average values $\alpha = 0.532$ and $pK' = 6.10$ were used.

SUMMARY

The solubility coefficient of CO_2 in twenty-two human urines was found to average 0.522 ± 0.08 at 38°. The maximum range of variation was from 0.498 to 536. The variation was chiefly a function of the salt concentration.

The value of Hasselbalch's constant, pK′, in human urine was found to average 6.10 ± 0.07. The maximum range of variation was from 5.97 to 6.21. This variation was also found to be chiefly a function of the salt concentration, according to the approximate equation, $pK' = 6.32 - 0.6\sqrt{B}$, where B represents equivalents of total base per liter of urine. The value of pK′, in solutions of the ionic strengths encountered in urine, was found to be changed by only 0.01 by addition of urea in the highest concentration (0.5 M) found in human urine.

The use of average α and pK′ values in calculation of urine pH from CO_2 tension and CO_2 content yielded pH values with an average error of ±0.03 and a maximum error of 0.10.

A line chart for urine has been constructed, with scales for CO_2 content, CO_2 tension, and pH, whereby any one of these variables can be calculated from known values of the other two.

BIBLIOGRAPHY

Fiske, C. H., *J. Biol. Chem.*, **51**, 55 (1922).

Gamble, J. L., *J. Biol. Chem.*, **51**, 295 (1922).

Hasselbalch, K. A., *Biochem. Z.*, **78**, 112 (1916).

Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **65**, 445 (1925).

Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, **65**, 381 (1925).

Hastings, A. B., Sendroy, J., Jr., and Van Slyke, D. D., *J. Biol. Chem.*, **79**, 183 (1928).

Henderson, L. J., *J. Biol. Chem.*, **7**, 29 (1909–10).

Hoffman, W. S., *J. Biol. Chem.*, **93**, 787 (1931).

Mainzer, F., and Bruhn, M., *Biochem. Z.*, **230**, 395 (1931).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **2** (1932).

Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *Sunti communicaz. scient., XIV cong. internaz. fisiol.*, Rome, 232 (1932).

Usher, F. L., *J. Chem. Soc.*, **97**, 66 (1910).

Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).

Van Slyke, D. D., and Sendroy, J., Jr., *J. Biol. Chem.*, **73**, 127 (1927); **79**, 781 (1928).

Van Slyke, D. D., Sendroy, J., Jr., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, **78**, 765 (1928).

Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **96**, 531 (1932).

STUDIES OF ACIDOSIS

XXIII. THE CARBON DIOXIDE TENSION AND ACID-BASE BALANCE OF HUMAN URINE*

By JULIUS SENDROY, Jr., S. SEELIG,† and DONALD D. VAN SLYKE

(*From the Hospital of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 15, 1934)

Marshall (1922) and Gamble (1922) were the first to correlate CO_2 content and pH in urine. They agreed in finding that the CO_2 content increased with the pH, urines of high alkalinity showing a bicarbonate content up to several grams per liter. Gamble further calculated the free carbonic acid, customarily symbolized as H_2CO_3, from observed pH and CO_2 content of the urine by means of the Henderson-Hasselbalch equation. He assumed for pK′ a value of 6.10, which had previously been found for blood plasma, and which in fact we now find to be the average value for urine also (Sendroy, Seelig, and Van Slyke, 1934). Urine CO_2 tension values calculated from Gamble's H_2CO_3 results, with Sendroy, Seelig, and Van Slyke's average value of 0.522 for α, indicate a variation from 51 to 76 mm., with an average of 61 mm.

Mainzer, with his collaborators (1929, 1931), has since studied the problem further. Like Gamble and Marshall, he measured directly, in the freshly collected urine, only the pH and total CO_2 content, and calculated the CO_2 tension from these values by means of the Henderson-Hasselbalch equation. In the second paper, Mainzer and Bruhn (1931) measured the pH with the quinhydrone electrode, and for each sample of urine they made separate determinations of pK′ and α for use in the calculation. The extent of the variations in pK′ and α encountered in different urine samples has already been indicated in the preceding paper (Sendroy, Seelig, and Van Slyke, 1934), in which we have compared Mainzer and Bruhn's results with our own. Mainzer and Bruhn's calculated CO_2 tensions in freshly voided urine varied between 13 and 242 mm., over an immensely wider range than those of Gamble. Mainzer concluded that there was no relationship between CO_2 tension in urine and CO_2 tension in blood.

---

* A preliminary communication concerning this work was delivered at the XIVth International Physiological Congress at Rome (Sendroy, Seelig, and Van Slyke, 1932).

† Fellow of the Rockefeller Foundation on leave of absence from the Charité Krankenhaus, Berlin, Germany.

The wide discrepancy between the urinary CO_2 tension ranges reported by Mainzer and those calculable from Gamble's data made a reexamination of the problem desirable. These authors did not make direct measurements of the CO_2 tension, but calculated it by the Henderson-Hasselbalch equation from the pH and total CO_2 content. In such calculations the combined errors in pH and CO_2 content determinations, and in the values for pK′ and α used in the Hasselbalch equation, all accumulate on the CO_2 tension.

In order to avoid such cumulative errors we have returned to direct measurement of the CO_2 tension by the tonometric procedure, introduced by Pflüger (1872) for estimating the gas tensions in blood, and applied to urine by Strassburg (1872). A relatively large volume of urine is equilibrated with a relatively small volume of gas until the latter attains the CO_2 tension of the urine. The tension is then ascertained by analysis of the gas.

Van Slyke, Sendroy, and Liu (1932) have applied this principle to the direct measurement of p_{CO_2} in blood,[1] and the technique used for urine is an adaptation of the same procedure. Since most samples of urine are low in CO_2 concentration, and many may be also low in buffer value, and because the extent of p_{CO_2} variation in urine is much greater than in blood, the ratio of gas volume to liquid volume has been changed from 1:9 for blood to 1:50 for urine. It has also been found necessary to prolong the equilibration time to ½ hour.

PROCEDURE

Method for Direct Measurement of Urine CO_2 Tension

Preparation of Gas Mixture—It is desirable that the gas mixture used have a CO_2 tension within 25 mm. of the urinary p_{CO_2}. One can calculate that if 50 volumes of buffer-free water, containing CO_2, only in the form of free carbonic acid, are equilibrated with 1 volume of gas, 25 mm. difference between gas and water with respect to initial p_{CO_2} will produce in the equilibrated system a final p_{CO_2} which differs by 1 mm. from the initial p_{CO_2} of the water. The gas tension will move 24 mm. towards the water tension, and the latter will move 1 mm. towards the initial gas tension. If the water contains buffers active at its pH, the final p_{CO_2} will be less than 1 mm. from the initial p_{CO_2} of the solution. Urine does contain such buffers, notably bicarbonate and phosphate, so that the correction to the final p_{CO_2} for initial difference in p_{CO_2} between urine and gas bubble is less than one-twenty-fifth of that difference. However, the buffer content of urine varies so greatly that it is impossible to estimate a routine correction for initial p_{CO_2} differences between urine and the gas bubble. One can only state that the error from failure to make such correction will be less than 1 mm. if the initial difference in p_{CO_2} between urine and gas bubble does not exceed 25 mm.

---

[1] The symbol p_{CO_2} is used to indicate CO_2 tension in mm. of mercury.

Accordingly, in our procedure the urine is equilibrated with an air-CO_2 mixture having a p_{CO_2} of 67 mm., the average p_{CO_2} of urine. This is within 25 mm. of the p_{CO_2} of the great majority of urines. If the final p_{CO_2} found by analysis of the equilibrated bubble is outside the range 67 ± 25 mm., a second portion of urine is equilibrated with gas that has a CO_2 tension approximating that found for the urine by the preliminary estimation. Since urinary p_{CO_2} is practically never below 40 mm., a second equilibration will be necessary only for urines with unusually high tensions. In such cases the urine is likely to be highly buffered, so that the error in the preliminary determination is usually not significant; but in order to make certain of accuracy, a second equilibration with gas containing more CO_2 is desirable.

For routine use, it is convenient to have on hand large amounts of the air-CO_2 mixture with 67 mm. p_{CO_2}. The percentage of CO_2 in such a gas mixture is calculated as $67/(0.01\ (B - 50))$, where B is the average barometric pressure. At sea level the CO_2 content of the desired mixture is 9.5 per cent. Our gas mixtures were prepared and stored in 300 cc. containers as described by Van Slyke, Sendroy, and Liu (1932, p. 554).

The other CO_2-air mixtures occasionally required for urines with higher CO_2 tensions may be prepared by mixing measured volumes of air and CO_2 in a gas burette. In our experiments, however, we prepared such mixtures by attaching the tonometer, before the urine was placed in it, directly to the gas manifold. The system was partially exhausted and the required pressure of CO_2 was added, measured by a manometer, as described on p. 302 of Peters and Van Slyke (1932).

Tonometer—The tonometer in which the equilibration takes place resembles that described by Van Slyke, Sendroy, and Liu, except that the body has a capacity of 50 cc. instead of 9 cc. for the liquid phase. The gas bubble volume is 1 cc.

Equilibration of Urine and Gas at 38°—The tonometer is first filled with the desired gas mixture. Enough urine ($>$100 cc.) for duplicate p_{CO_2} determinations is voided under oil, and transferred at once to a container over mercury, from which it is introduced into the tonometer to fill all but 1 cc. of its volume. Urine and gas are equilibrated at 38° as described by Van Slyke, Sendroy, and Liu (1932) for blood samples, except that the length of time required for complete equilibration is extended to ½ hour. The vessel is opened to the air after 5, 10, and 15 minute periods to keep the pressure at atmospheric, and is then rotated for another 15 minutes. Satisfactory results have been obtained with some samples of urine rotated for a shorter time, but for uniformly good results, for widely different samples of urine, 30 minutes have been found necessary.

Analysis of Gas Bubble—This is carried out as described by Van Slyke, Sendroy, and Liu (1932). The gas sample measurement by manometric pressure is made with the gas at 2.0 cc. volume. The pressure of the CO_2 gas is usually measured at 0.5 cc. volume. However, some samples of urine high in CO_2 tension yield so much CO_2 to the bubble, that measurement of the CO_2 pressure must be made with the extracted CO_2 at 2.0 cc. volume.

Calculation

The CO_2 tension is calculated from the volume per cent CO_2 content, *C*, of the gas bubble, according to the usual equation

$$p_{CO_2} = 0.01\ C\ (B - 49.7) \qquad (1)$$

where B is the barometric pressure in mm. of mercury at the time of the gas bubble equilibration, and 49.7 is the vapor tension of water at 38°.

EXPERIMENTAL

Comparison of Known Urine CO_2 Tensions with Tensions Found by Present Method

Urine samples of about 110 cc. were saturated at 38° with known mixtures of CO_2 and air, in a two-chambered saturating vessel by the "First saturation method" of Austin *et al.* (1922). After complete gaseous equilibrium had been attained, the gas and liquid phases were separated, and the gas was analyzed for CO_2 in a Haldane apparatus. From the CO_2 content of the gas, the CO_2 tension of it, and therefore of the urine, was calculated with an error not exceeding 0.2 mm. The tensions thus obtained are given in Table I as "CO_2 tension of urine at 38°, present." The urine was then used for duplicate determinations of the CO_2 tension by the present method.

The results, recorded in Table I, indicate that the error of urine CO_2 tensions measured as described in this paper averaged 1.1 mm. The maximum error found, occurring in but one case, was 3.1 mm.

In this series of experiments, the maximum difference between the initial p_{CO_2} of the bubble and the true p_{CO_2} of the urine was 31 mm. It does not appear from the results that initial differences within this limit have a significant effect on the final p_{CO_2} determined.

CO_2 Tension of Freshly Voided Human Urine

60 individual samples of urine were analyzed by the procedure for direct p_{CO_2} presented in this paper. The urines were taken at intervals varying from 1 to 10 hours after previous voiding, and under various conditions of activity. Each sample was collected at a single voiding.

For the first analysis in each case the initial tension of the bubble was set at 67 mm. The second analysis was performed with p_{CO_2} of the bubble approximating the value obtained by the first analysis. When the urine p_{CO_2} was over 100 mm. the result of the second analysis was taken as the p_{CO_2} value. Except when values above 130 mm. were encountered, the agreement between the two analyses was within the limit of error of the method.

Other data recorded for each specimen were specific gravity, pH, and the period of urine retention for the particular voiding analyzed. The specific gravity measurements were made with an ordinary urine specific gravity bulb; their maximum error is about 0.003. The pH values were obtained colorimetrically according to the method of Hastings, Sendroy, and Robson (1925) but with the use of a Hastings-Duboscq colorimeter in place of the acid-alkali standard color tubes (Peters and Van Slyke, 1932, p. 801).

TABLE I

Urine CO_2 Tensions Found by Present Method Compared with Tensions Set by Saturation with Known CO_2-Air Mixtures

Urine No.	Initial CO_2 tension of gas bubble at 38°	CO_2 tension of urine at 38°		
		Present	Found from final p_{CO_2} of gas bubble	Error
	mm.	*mm.*	*mm.*	*mm.*
1	82	90.6	89.2	−1.4
2	82	98.2	99.6	+1.4
3	82	95.9	94.4	−1.5
4	82	104.9	105.3	+0.4
5	82	98.6	97.1	−1.5
6	82	99.9	97.8	−2.1
7	82	96.0	96.5	+0.5
8	82	97.8	97.3	−0.5
9	82	99.6	96.5	−3.1
10	82	99.3	98.9	−0.4
11	82	99.2	99.1	−0.1
12	82	67.9	69.1	+1.2
13	79	62.7	61.0	−1.7
14	82	55.7	56.2	+0.5
15	58	32.7	34.3	+1.6
16	43	23.4	23.4	±0.0
17	172	140.7	139.7	−1.0
18	172	159.5	158.6	−0.9
19	96	80.1	81.1	+1.0
20	96	75.4	76.5	+1.1
Average				±1.1

The urine specimens examined fell into three groups: normal, pathological, and experimental. The first group consisted of thirty specimens obtained from normal individuals under ordinary conditions. The second group consisted of fourteen specimens obtained from patients at this hospital suffering from nephritis or cardiac disease. The third group of sixteen specimens was obtained from normal individuals whose acid-base balance was purposely shifted from normal by the administration of alkali or by exercise.

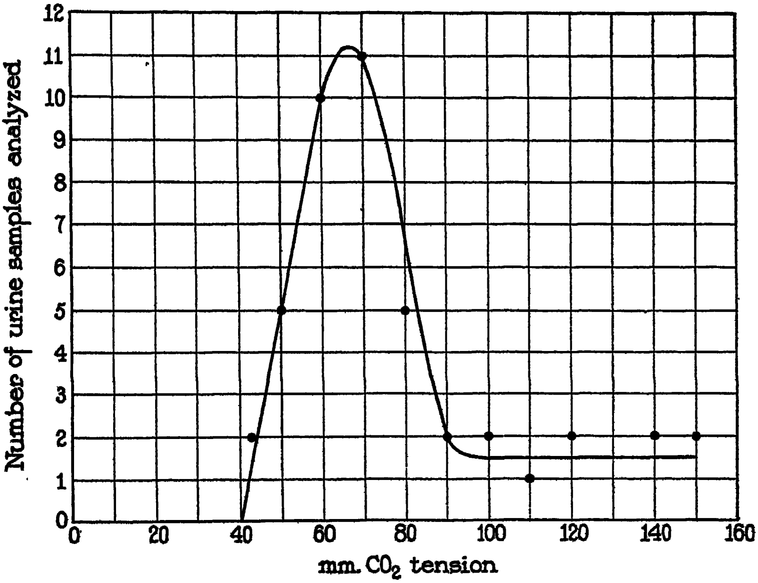


Fig. 1. Distribution curve of normal and pathological human urine CO_2 tension results at 38°.

RESULTS

In Fig. 1 is given the distribution curve for urine p_{CO_2} results found in our study of forty-four normal and pathological cases. The urine p_{CO_2} of the normal individuals varied from 53 to 149 mm.

The variation in pathological cases covered nearly the same range, from 42 to 124 mm.

Fig. 1 indicates that in our study there are more naturally occurring urine CO_2 tensions at 67 ± 5 mm. than at any other level, 25 per cent of the results being at that level. Furthermore, 77 per cent of the results are between 42 and 95 mm. The curve is peculiar in that there is no asymptotic approach to the abscissa on either end. The lower end of the curve cuts the abscissa at a sharp angle, since no results were found for urine p_{CO_2} below 40 mm. The upper end of the curve runs from 95 mm. to 149 mm. parallel to the abscissa.

TABLE II

CO_2 Tension in Urine after Experimental Alteration of Individual Acid-Base Balance

Urine No.	Specific gravity	Period	CO_2 tension of urine by bubble analysis	pH	Treatment
		hrs.	*mm.*		
1	1.024	10¼	119	7.4	23 gm. $NaHCO_3$
2	1.008	2	42	6.3	H_2O drinking
3	1.018	3	124	6.5	5 gm. Na citrate
4	1.023	3¼	169	5.8	Exercise, playing squash
5	1.025	3¼	159		" stair-running
6	1.032	3⅔	136	7.2	5 gm. $NaHCO_3$ + stair-running
7	1.015	4½	58	5.3	Stair-running
8	1.026	1¾	183	5.1	Exercise, stair-running
9	1.026	2½	70	5.5	5 gm. $NaHCO_3$ + stair-running
10	1.009	2	55	6.0	Exercise, stair-running
11	1.010	2	73	5.5	5 gm. $NaHCO_3$ + stair-running
12	1.007	2¼	51	6.1	Exercise, stair-running
13	1.019	3	57	5.4	HCl + stair-running
14	1.032	3	52	5.0	Exercise
15	1.035	2½	201	7.5	5 gm. $NaHCO_3$ + stair-running
16	1.024	2¼	177	4.9	Exercise, stair-running

When the sixteen experimental cases recorded in Table II are also considered, there is found little to alter this picture. The lower limit is 42 mm. for urine p_{CO_2} obtained after dilution by drinking large amounts of water (Urine 2). The upper limit is 201 mm. obtained after the ingestion of 5 grams of $NaHCO_3$ and severe exercise (stair-running, 30 meters up and down, three times, Urine 15). Indeed, *all of the five results higher than 150 mm.*

among our total number of 60 urine sample determinations *were obtained after the subject had exercised.* Results between 100 and 150 mm. were usually obtained after the ingestion of alkali in the form of citrate or bicarbonate. A few high results were also obtained in apparently normal subjects under ordinary conditions.

Relationship between Urine CO_2 Tension and Specific Gravity

Fig. 2 indicates that none of our urine samples with specific gravity lower than 1.018 was of a CO_2 tension higher than 80 mm. Higher CO_2 tensions occurred only in fairly concentrated urines.

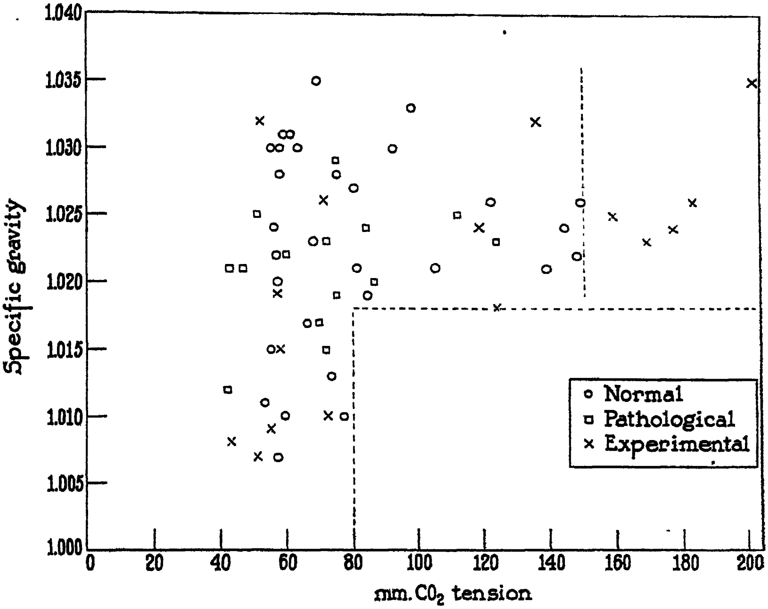


FIG. 2. The relation between CO_2 tension of urine and the specific gravity

Relationship between Urine CO_2 Tension and Period of Accumulation in the Bladder

Fig. 3 shows that no urine retained over 4 hours had a CO_2 tension over 120 mm., while a third of those retained less than 4 hours had tensions over 120 mm., ranging up to 200 mm. Apparently, high CO_2 tensions are to be found only in concentrated urines stored in the bladder for a short period, but the converse of this is not true; namely, that high concentration or short period of accumulation, or both, result uniformly in high CO_2 tensions.

A possible explanation for the absence of high CO_2 tensions (greater than 120 mm.) in urine retained over 4 hours is that pro-

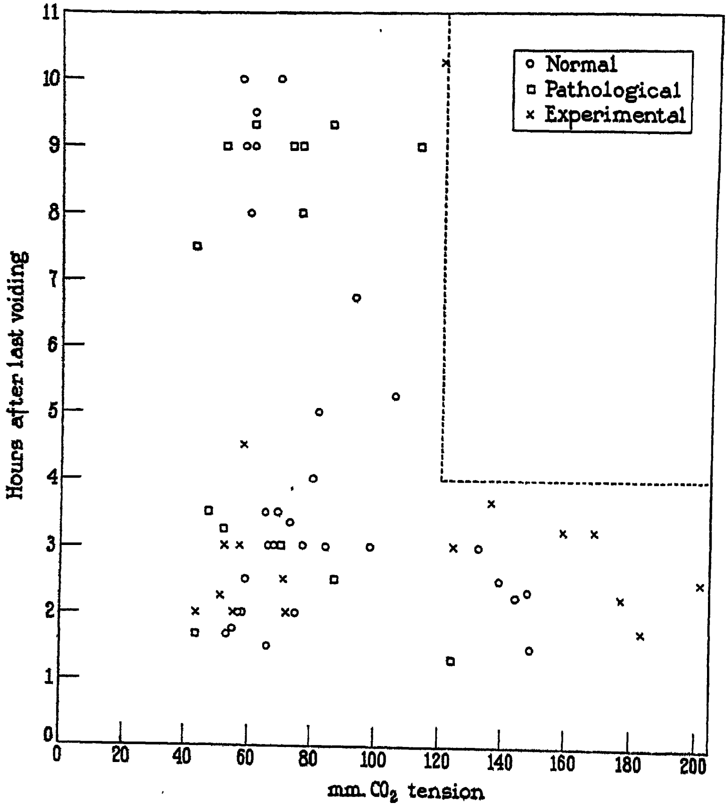


FIG. 3. The relation between CO_2 tension of urine and the period of accumulation in the bladder.

longed accumulation in itself tends to produce a urine of average composition. If temporary conditions produce a urine of high CO_2 tension during 1 hour, it may be diluted with urine of usual tension during subsequent hours. In the one observation providing an exception to the 4 hour rule (Urine 1, Table II), an abnormal state was prolonged by taking 15 grams of $NaHCO_3$ in the evening and 8 grams more in the morning before voiding. The urine, voided after 10 hours collection, had a high CO_2 tension.

An alternative explanation is that free carbonic acid, which is one of the most diffusible of physiological solutes, slowly diffuses through the bladder wall when the CO_2 tension of the urine is higher than that of the blood.

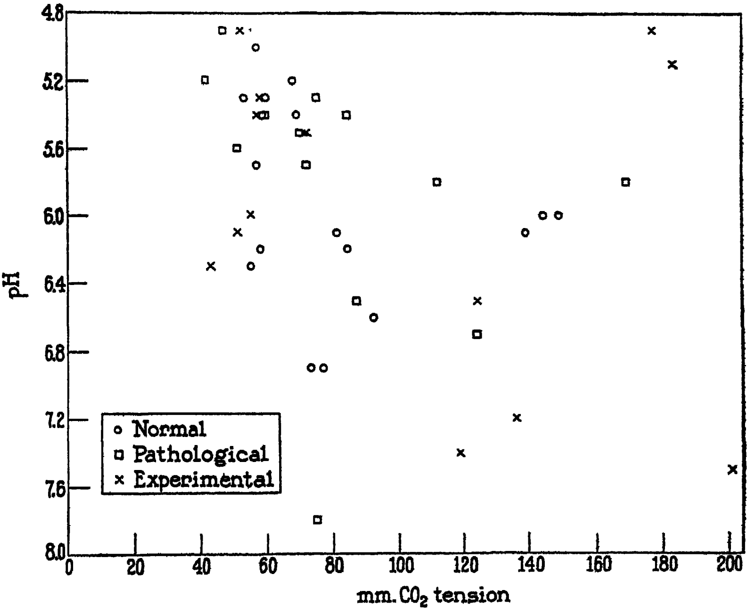


Fig. 4. The relation between CO_2 tension and pH of urine

In general, one may say that dilute urines, or urines stored in the bladder for a long period, tend to approach the average value of 67 mm. for CO_2 tension (see upper left corner of Fig. 3, and lower left corner of Fig. 2). Herein may lie the explanation for Gamble's (1922) comparatively constant urinary H_2CO_3 values.

Absence of Relationship between CO_2 Tension and pH

As is indicated by Fig. 4, we have found in agreement with Mainzer and Bruhn (1931), no correlation between the pH and the CO_2 tension of urine.

TABLE III

Summary of Urine CO_2 Tension Values Recorded in Literature

Author	Method	Urine samples		CO₂ tension			Remarks
		No. analyzed	Species	Variation	Mean	Distribution	
				mm.	*mm.*		
Strassburg (1872)............	Direct, tonometric	3	Dog	56– 84	70		Fresh samples
Fredericq (1910)............	Direct, tonometric	43	Human	48–137	77		" "
		6	Dog	50–100	84		
Gamble (1922)...............	Calculated from CO_2 and pH	55	Human	48– 76		80%, 55–75 mm.	" "
Mainzer and Shen (1929).....	By interpolation on CO_2 absorption curves	7	"	43–102	81	All but two higher than 86 mm.	" "
" " Bruhn (1931)...	Calculated from CO_2 and pH with individual α and pK′ values	20	"	13–242		Only four (13, 17, 35, and 39 mm.) below 40 mm.	" " all subjects with $NaHCO_3$ or H_2O administered
Present authors (1934).......	Direct, tonometric	30	" normal	53–149		25% at 67 mm., 80%, 42–95 mm.	
		14	" pathological	42–124			
		16	" experimental	43–201			

Urine CO_2 Tensions Found by Other Authors

In Table III we have summarized the results available in the literature. The data of Strassburg (1872), Fredericq (1910), and our own are the only ones obtained by direct tonometric methods. Fredericq's results for forty-three human urine p_{CO_2} values, from 48 to 137 mm., agree well with our forty-four values from 42 to 149 mm. The other data have been sufficiently discussed in the introduction.

Acid-Base Balance of Freshly Voided Human Urine

With the results of this and of the preceding paper (Sendroy, Seelig, and Van Slyke, 1934) we are able to estimate for twenty-one freshly voided human urines the acid-base balance, in the terms of pH, $[CO_2]$, and p_{CO_2} commonly used to define the acid-base balance of the blood. With the individual α and pK′ values found for each urine, the pH was calculated according to the Henderson-Hasselbalch equation

$$\text{(2)} \qquad \text{pH} = \text{pK}' + \log \frac{[CO_2] - 0.1316\,\alpha\,p_{CO_2}}{0.1316\,\alpha\,p_{CO_2}}$$

with CO_2 content in terms of volume per cent.

The results are contained in Table IV of this paper, and are plotted in Fig. 5 on the logarithmic chart used by Peters and Van Slyke ((1931) p. 915). Fig. 5 is convenient for charting acid-base data in fields showing normal and abnormal variations. For its construction, the average values of $\alpha = 0.522$ and $\text{pK}' = 6.10$ were used in Equation 2 above, or, more conveniently, in the transformed equation

$$\text{(3)} \qquad [CO_2] = 0.1316\,\alpha\,(10^{\text{pH}-\text{pK}'} + 1)\,p_{CO_2}$$

The magnitude of error introduced by using average α and pK′ values is indicated in the preceding paper (Sendroy, Seelig, and Van Slyke, 1934).

In addition to the results of Table IV, we have also plotted the positions, in Fig. 5, of other urine samples, for which we have determined direct p_{CO_2} and colorimetric pH values. Included in the same chart are the results of Marshall (1922) from CO_2 content and colorimetric pH values, those of Gamble (1922) from CO_2 content and calculated p_{CO_2}, and those of Mainzer and Bruhn (1931) likewise from CO_2 content and calculated p_{CO_2} values.

There is rather good agreement between our results and those of Mainzer and Bruhn, in that there is a similarly wide scattering of points outside of the area in which the majority of urines fall. This majority area may be considered as bounded by the limits

TABLE IV

Acid-Base Balance of Freshly Voided Urine at 38°

Urine No.	αCO_2 determined	pK′ determined	CO_2 tension determined	CO_2 content determined		pH calculated by Equation 2
	cc. CO_2 per cc. solution		*mm.*	*mM per l.*	*vol. per cent*	
1	0.524	6.21	56.8	1.928	4.292	5.20
2	0.504	6.05	119.0	75.37	167.77	7.36
3	0.536	6.14	41.6	3.360	7.479	6.33
4	0.503	6.00	59.4	2.115	4.708	5.29
5	0.498	6.04	68.8	2.481	5.523	5.39
6	0.524	6.07	124.3	16.90	37.62	6.60
7	0.528	6.15	52.6	1.898	4.225	5.35
8	0.523	6.11	41.5	1.441	3.208	5.20
9	0.531	6.09	124.5	18.20	40.51	6.65
10	0.515	6.16	69.8	2.595	5.776	5.51
11	0.532	6.15	54.6	3.016	6.714	6.04
12	0.520	6.08	73.0	18.28	40.69	6.94
13	0.530	5.97	51.1	2.810	6.255	6.09
14	0.529	6.14	56.9	2.127	4.735	5.43
15	0.527	6.11	76.8	16.22	36.11	6.87
16	0.518	6.06	84.0	6.01	13.28	6.18
17	0.530	6.13	51.0	2.110	4.687	5.64
18	0.522	6.12	74.6	2.621	5.834	5.26
20	0.530	6.13	86.2	9.75	21.70	6.54
21	0.513	6.03	69.6	2.724	6.064	5.49
22	0.532	6.18	59.1	2.188	4.870	5.43

pH 5.0 to 6.4, p_{CO_2} 40 to 85 mm., and CO_2 content 3 to 13 volumes per cent, or 1.3 to 6 mM per liter.

Compatibility of Results with Filtration-Reabsorption Theory of Urine Secretion

The recent work of Richards (quoted below) and of Smith and his collaborators (Jolliffe, Shannon, and Smith, 1932; Shannon,

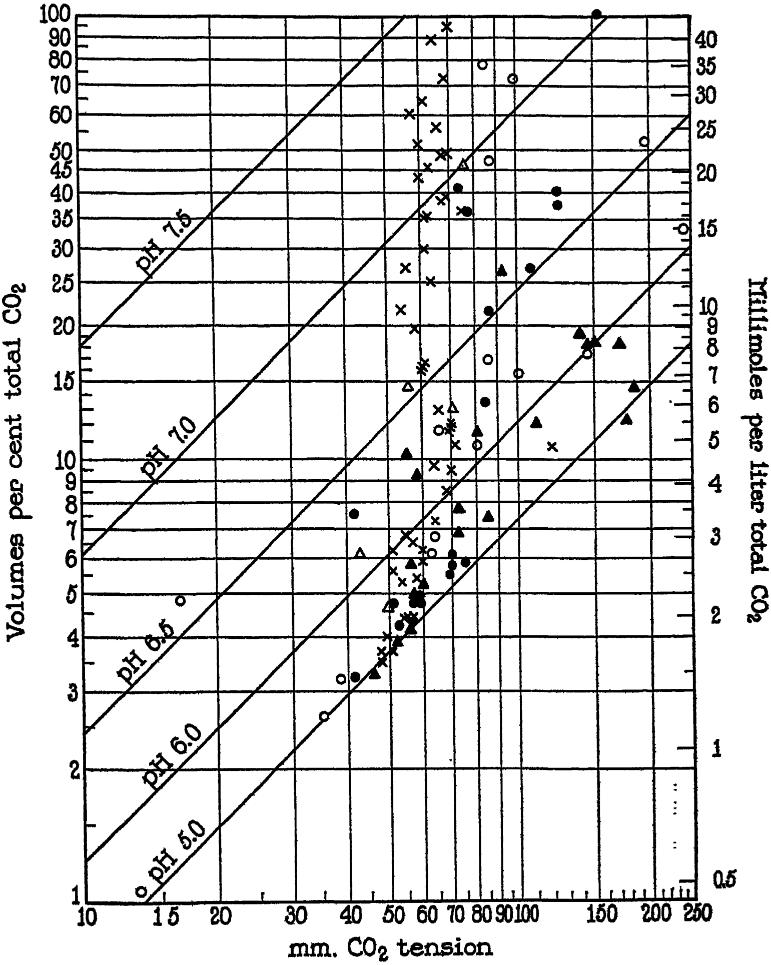


FIG. 5. Acid-base balance results for human urine. ○ Mainzer and Bruhn (1931), from CO_2 content and tension; × Gamble (1922), from CO_2 content and tension; △ Marshall (1922), from CO_2 content and pH; ● authors (1934), from CO_2 content and tension; ▲ authors (1934), from CO_2 content and pH.

Jolliffe, and Smith, 1932) has provided strong experimental support for this theory, and it appears of interest to ascertain whether the present results concerning the acid-base balance of the urine are in accord with it. As the simplest way in which to demonstrate such agreement we outline the sequence of events which, according to the theory, lead to the production of urine showing the demonstrated relationships of $BHCO_3$, H_2CO_3, and pH. The explanation outlined is tentative, but to avoid repeated reservations, the events are sketched as though the filtration-reabsorption mechanism were proved.

Formation of Acid Urine.—In the glomerular filtrate H_2CO_3 and $BHCO_3$ are presumably present in approximately the same concentrations as in the blood plasma. It is true that these concentrations of H_2CO_3 and $BHCO_3$ have not been analytically demonstrated in the filtrate, but Wearn and Richards (1924) have shown that the filtrate has the same pH as the plasma, so that the ratio, $H_2CO_3:BHCO_3$, is the same as in the plasma. Furthermore, when the other crystalloids, Cl, urea, uric acid, glucose, creatinine, PO_4, are filtered with unchanged concentrations, as shown by Richards and his collaborators, it appears reasonable to assume that H_2CO_3 and $BHCO_3$ are similarly filtered.

As the filtrate proceeds down the tubules it ordinarily becomes more acid, as has been colorimetrically demonstrated in the frog kidney by Richards (1929). It appears probable that the *acidification is the result of active reabsorption of* $BHCO_3$ in the same way that BCl is reabsorbed, the tubular cells returning both BCl and $BHCO_3$ from the filtrate to the blood as completely as may be necessary to maintain the normal supply of these salts in the body. In the cases of both chloride and bicarbonate, reabsorption may be so complete that only traces remain in the urine. In human urine, of the average normal pH of 6.1, the $BHCO_3$ concentration is only about one-tenth as great as in the blood plasma (Fig. 5). Table V illustrates, by average values, the estimated amounts of $BHCO_3$, H_2CO_3, and PO_4 which are passed into the glomerular filtrate, and the amounts which escape reabsorption and appear in the urine.

If one assumes that the glomerular filtrate forms in accordance with the filtration-reabsorption theory, and in the amounts indicated by either the xylose or the creatinine clearances (Jolliffe.

Shannon, and Smith, 1932; Shannon, Jolliffe, and Smith, 1932), it appears difficult to explain the smallness of the amounts of $BHCO_3$ found in acid urine by any process other than tubular reabsorption. Calculated as $NaHCO_3$, the amount of bicarbonate filtered in the 24 hour glomerular filtrate of about 150 liters would be about 340 grams, containing 92 grams of Na, while in the daily output of urine only 3 or 4 grams of Na are ordinarily found, chiefly in the form of chloride, and only a fraction of a gram of alkali bicarbonate (Table V).

TABLE V

Estimated 24 Hour Filtration and Excretion of $BHCO_3$, H_2CO_3, and PO_4 by Human Kidney

Substance	Glomerular filtrate,* 150 liters at pH = 7.4		Urine, 1.5 liters at pH = 6.1		Urine, 1.5 liters at pH = 5.0	
	Concentration	Total amount	Concentration	Total amount	Concentration	Total amount
	mM per l.	*mM*	*mM per l.*	*mM*	*mM per l.*	*mM*
$BHCO_3$..............	25	3700	2	3	0.1	0.15
H_2CO_3..............	1.3	195	2	3	1.3	2
PO_4..................	1	150	43	65	43	65

* From the results of Jolliffe, Shannon, and Smith (1932) it is assumed that the glomerular filtrate is about 1.4 times the maximum urea clearance. Møller, McIntosh, and Van Slyke (1928) have found the maximum urea clearance to average 75 cc. per minute in normal men. Hence the glomerular filtrate is calculated to be 1.4 × 75 = 10.5 cc. per minute, and 1440 × 0.104 = 149 liters per 24 hours. The 65 mM of PO_4 excretion (= 2 gm. of P) is the maximum likely to be found in the human 24 hour urine.

Free H_2CO_3, unlike $BHCO_3$, appears not to be "actively" reabsorbed, but merely to pass back by diffusion into the blood with reabsorbed water. There is no physiological need for H_2CO_3 reabsorption, since CO_2 is continually produced by combustion in the body. Evidence that H_2CO_3 reabsorption is never "active" in the sense that the concentration in the tubular lumina is lowered below that in the blood, is seen in the fact that the CO_2 tension in the urine never, according to our results, is found below that in the arterial blood.

The changes in $BHCO_3$ and H_2CO_3 which acidify the urine must also affect the acid-salt ratios of other buffers. Of these the most important are normally the phosphates (Peters and Van Slyke, 1931, pp. 959 and 1111). With alkaline phosphate the reversible reaction

$$(4) \qquad H_2CO_3 + B_2HPO_4 \rightleftarrows BHCO_3 + BH_2PO_4{}^*$$

must occur until the relationship indicated by Equation 5 is established.

$$(5) \qquad \frac{[B_2HPO_4]}{[BH_2PO_4]} = 0.2\,\frac{[BHCO_3]}{[H_2CO_3]}$$

(The factor 0.2 is the approximate ratio of the second dissociation constant of H_3PO_4 to the first dissociation constant of H_2CO_3.)

It appears that all the phosphate excreted can be changed in the tubules from B_2HPO_4 to BH_2PO_4 by the reaction of Equation 4 if the following conditions are met: (1) The $BHCO_3$ formed by the reaction must be absorbed, so that the reaction can continue from left to right. (2) At least 1 mole of H_2CO_3 per mole of B_2HPO_4 must be present in the initial glomerular filtrate. (3) The H_2CO_3 in the filtrate must not be diminished (by absorption) below this proportion until the reaction and the reabsorption of $BHCO_3$ are completed. It appears possible that all these conditions may be met. (1) As indicated by Table V, reabsorption of $BHCO_3$ in urine of ordinary acidity is nearly complete. (2) There is more than 1 mole of H_2CO_3 per mole of B_2HPO_4 present in the calculated filtrate. (3) Concerning the relative reabsorption velocities of H_2CO_3 and $BHCO_3$ there are no data. Since about 20 times as many moles of $BHCO_3$ as of H_2CO_3 are reabsorbed, however, it appears quite possible that reabsorption of $BHCO_3$ may sufficiently outrun that of H_2CO_3 to meet the third condition.

The acidifying effect of $BHCO_3$ reabsorption may be reenforced by B_2HPO_4 reabsorption, since only part of the glomerular PO_4 reaches the urine (Table V). However, to shift the B_2HPO_4: BH_2PO_4 ratio of 4:1 in the glomerular filtrate to the value of practically 0 in urine of pH 5, by B_2HPO_4 absorption alone, would require reabsorption of 80 per cent of the filtrate PO_4 (all the B_2HPO_4). It does not appear from Table V that phosphate reabsorption always proceeds to this extent. Hence, at times, in any case, part of the decrease in the $B_2HPO_4:BH_2PO_4$ ratio, which occurs as the filtrate passes down the tubules, is apparently due to other acidifying causes, such as $BHCO_3$ reabsorption and the consequent reactions. Under no condition could B_2HPO_4 reabsorption, by reversal of the reaction (Equation 4), have much effect in decomposing filtrate $BHCO_3$, because the amount of the latter is so immensely greater.

---

* This reaction, with tubular absorption of the $BHCO_3$, was originally proposed by Cushny (1926) as a factor in acidification of urine.

If the change from the alkaline to the acid form of phosphate during passage of the tubules is accomplished by reaction of the alkaline form with H_2CO_3 (Equation 4) and reabsorption of the $BHCO_3$ formed, a similar process must occur with the other urinary buffers. The condition under which the greatest demand is put upon such a mechanism is presumably that of severe diabetic acidosis, in which large amounts of free hydroxybutyric acid are added to the acid phosphate in the urine. However, the maximum amount of total titratable acid (free organic acid plus acid phosphate) found by Stillman, Van Slyke, Cullen, and Fitz (1917) in severe diabetic acidosis was 120 mM per 24 hours. This is not beyond the possibility of production by reaction of sodium hydroxybutyrate with the 195 mM of H_2CO_3 calculated in Table V to be present in the glomerular filtrate, the resultant $BHCO_3$ being reabsorbed, and the free organic acid left in the urine. Acidification by reabsorption of alkali salts of hydroxybutyric acid can hardly be important, because, to lower the pH to 5 by this means would require reabsorption of about 98 per cent of the hydroxybutyrate in the glomerular filtrate, and the large amounts excreted (up to 75 grams in the 24 hour urine) preclude such complete reabsorption.

Besides reabsorption of $BHCO_3$ and B_2HPO_4, an alternative method of acidifying the urine, and preserving the alkali reserve of the body, would be the excretion of free HCl into the tubular lumina (no other anion than Cl^- in the normal blood could provide sufficient acid to react with the amount of $BHCO_3$ estimated to be in the glomerular filtrate). Similarly one could assume in diabetic acidosis the tubular excretion of free hydroxybutyric acid. However, the fact that the pH of the urine, unlike that of the gastric juice, is never below 4.8, an acidity which could be produced by either H_2CO_3 or BH_2PO_4 left after reabsorption of $BHCO_3$ and B_2HPO_4, inclines one to favor the hypothesis that reabsorption of the alkaline buffer salts produces the acidification of the urine. (In a solution under 67 mm. of CO_2 tension, with $BHCO_3$, and hence B_2HPO_4 completely removed, the pH would be about 4.4.) Control of the relative speed and completeness with which $BHCO_3$ and H_2CO_3 are reabsorbed appears to be sufficient to control the entire observed range of urinary acidity and alkalinity.

The mechanism by which ammonia, formed in the kidney (Nash and Benedict, 1921), causes *excretion of acids as ammonium salts*, without loss of fixed base from the body, is also explicable by active reabsorption of $BHCO_3$. The excretion can be explained by assuming that NH_4HCO_3 formed in the kidney is filtered together with BA brought by the blood, and that in the tubules ions are interchanged, $BHCO_3$ is reabsorbed, and NH_4A is left in the urine. (A = anion of hydrochloric, hydroxybutyric, or other acid excreted as ammonium salt.)

Formation of Alkaline Urine—When plasma bicarbonate concentration is raised above a level of about 31 mM, the urine usually becomes alkaline (Palmer and Van Slyke, 1917). There is in such urine a relatively high concentration of $BHCO_3$ (see Gamble (1922); Marshall (1922); also data in this paper). Excretion of $NaHCO_3$ may reach several grams per hour. The glomerular filtrate of about 6 liters per hour, if also of more than 31 mM $NaHCO_3$ concentration, would contain over 20 grams of $NaHCO_3$ per hour. Hence the observed maximal urinary bicarbonate excretion, of at most a fraction of this amount, can be accounted for merely by incomplete reabsorption of the filtered $BHCO_3$. The relatively large concentrations of $BHCO_3$ in the urine are sufficient to cause the maximal observed pH values, about 8, and by interaction with the phosphates and other buffers, would increase the urinary ratios of their alkali salts to their free acids or acid salts.

SUMMARY

A technique is described for measurement of CO_2 tension in urine. The urine is equilibrated at 38° with 0.02 its volume of an air-CO_2 mixture, and the CO_2 tension is determined by microanalysis of the gas bubble.

The method has been checked by determinations on urines in which the CO_2 tensions were set at levels known within 0.2 mm.

The error of the method averaged 1 mm., and in no case exceeded 3 mm.

CO_2 tensions of human urines drawn from the bladder, in both normal and pathological cases, were found over the range from 40 to 200 mm. The majority were between 45 and 95 mm. The greatest accumulation was within the zone 67 ± 5 mm., within which one-fourth of the tensions was found.

CO_2 tensions above 80 mm. were found only in urines sufficiently concentrated to have specific gravities of 1.020 or higher. Tensions above 120 mm. were frequently found after severe exercise, and after taking sodium bicarbonate. Such high tensions, however, were not observed in any case in which the urine had been retained in the bladder longer than 4 hours.

The fact that urine tensions below 40 mm. were never encountered makes it appear doubtful that the CO_2 tension of the urine ever falls below that of the arterial blood.

Our results confirm Gamble's (1922) in showing that CO_2 tension and H_2CO_3 concentration in urine remain within the same range, regardless of variations in total CO_2 and $BHCO_3$ content, so that high $BHCO_3$ content tends to produce urine of high pH (Fig. 5). Our data differ from Gamble's only in showing, because of wider CO_2 tension range, somewhat less degree of correlation between $BHCO_3$ and pH.

The results are compatible with the filtration-reabsorption theory of urine excretion. The entire observed ranges of urinary CO_2 tension, CO_2 content, and pH can be quantitatively deduced from reasonably probable variations in reabsorption of $BHCO_3$ and H_2CO_3 from the glomerular filtrate.

BIBLIOGRAPHY

Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, **54**, 121 (1922).

Cushny, A. E., The secretion of urine, London, 206 (1926).

Fredericq, L., *Arch. internat. physiol.*, **10**, 391 (1910).

Gamble, J. L., *J. Biol. Chem.*, **51**, 295 (1922).

Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, **65**, 381 (1925).

Jolliffe, N., Shannon, J. A., and Smith, H. W., *Am. J. Physiol.*, **104**, 639 (1932).

Mainzer, F., and Bruhn, M., *Biochem. Z.*, **230**, 395 (1931).

Mainzer, F., and Shen, C. T., *Arch. ges. Physiol.*, **222**, 1 (1929).

Marshall, E. K., Jr., *J. Biol. Chem.*, **51**, 3 (1922).

Møller, E., McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Inv.*, **6**, 427 (1928).

Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, **48**, 463 (1921).

Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, **32**, 499 (1917).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Baltimore, **1** (1931); **2** (1932).

Pflüger, E., *Arch. ges. Physiol.*, **6**, 69 (1872).

Richards, A. N., Methods and results of direct investigations of the function of the kidney, Baltimore (1929).

Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *Sunti comunicaz. scient., XIV cong. internaz. fisiol.*, Rome 232 (1932); *J. Biol. Chem.*, **106**, 463 (1934).

Shannon, J. A., Jolliffe, N., and Smith, H. W., *Am. J. Physiol.*, **104**, 534 (1932).

Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, **30**, 405 (1917).

Strassburg, S., *Arch. ges. Physiol.*, **6**, 65 (1872).

Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **96**, 547 (1932).

Wearn, J. T., and Richards, A. N., *Am. J. Physiol.*, **71**, 209 (1924).

FURTHER STUDIES ON THE ZYMOGENS OF PEPSIN AND RENNIN

BY ISRAEL S. KLEINER AND HENRY TAUBER

(*From the Department of Physiology and Physiological Chemistry of the New York Homeopathic Medical College and Flower Hospital, New York*)

(Received for publication, June 14, 1934)

Recently we have reported additional evidence that (1, 2) pepsin and rennin exist in the mucosa of the stomach of the pig and calf respectively in the form of precursors or zymogens. We have found that the zymogens of pepsin and rennin differ in the pH values at which they become activated. There is some active rennin in neutral extracts of the mucosa of the fourth stomach of the calf, but this is not the case with similar extracts of the stomach of the pig. That the milk-coagulating power is a function of the pepsin molecule is now generally accepted (Hammarsten, Pekelharing, Fenger, Northrop, and others). The preformed activity of rennin we found to be separable from the inactive prorennin, if the neutral extract is kept at 37° for a few days, preserved with toluene, or if it is adjusted to pH 9 to 10. The active rennin is readily destroyed at an alkaline pH; the zymogen is not.

However, our findings, which point to a definite difference between propepsin and prorennin, have been contradicted by Ege and Lundsteen (3). For this reason we have taken up the study of this problem again in order to find the cause of the discrepancy.

EXPERIMENTAL

Preparation of Neutral Extracts—The same procedure as in our earlier experiments (1, 2) was employed; *i.e.*, to 75 gm. of washed and ground mucosa of fresh calf and pig stomach, respectively, 150 cc. of a 2 per cent $CaCO_3$ suspension in distilled water were added, stirred for 8 minutes, and filtered. The pH was 7.2 in the case of the extract of the calf mucosa and 7.1 in the case of the pig stomach extract. The pH measurements were made electrometrically. The Danish workers (3, 4) extracted *dried* stomachs with sodium phosphate solutions or with distilled water, which may have an acidity as high as pH 5.6 (5). As substrate they used a milk of much higher acidity (pH 5) than we had employed (pH 6.3).

Preparation of Prorennin Free of Rennet Activity—This can be obtained either by keeping a sample of neutral extract of prorennin with a heavy layer of toluene at 37° for about 3 to 4 days or by adjusting a sample of the fresh neutral extract to pH 9.2, keeping it for 40 minutes at 24°, and then neutralizing it carefully to pH 7. We found extreme care to be necessary to avoid an excess of acid while neutralizing; otherwise, activation of the inactive precursor might take place in local areas. The neutral calf extract showed a preformed activity of 10 to 20 per cent of the total potential activity; whereas the pig extract showed none when tested for 18 hours (milk of pH 6.3) with equal amounts of milk and extract. When milk of pH 5 was used, 1 cc. of the neutral pig extract had an activity of about 1/40,000 of the total potential activity, or 1 cc. of the neutral pig stomach extract could clot 0.4 cc. of milk at 37° in 10 minutes. These simple experiments of freeing prorennin solutions of preformed rennet activity could not be repeated by Ege and Lundsteen. It is possible that they did not exercise the necessary care in neutralizing the extract, thus activating the precursors inadvertently. Furthermore, they state that they could not find a difference between prorennin and propepsin in any other way, although Ege and Thygesen (4) in an earlier paper used an alkaline extraction of the pig stomach mucosa to free propepsin from pepsin, and Ege and Lundsteen's paper is an attempt at verification of the earlier work on the activation of propepsin from the same laboratory.

Difference in pH of Activation between Propepsin and Prorennin—Using as substrate milk having a pH of 5, Ege and Lundsteen ((3) p. 173) state that, at 40°, 30 minutes are required for 50 per cent activation of prorennin at pH 3.4. We have repeated this experiment. After the prorennin extract had been adjusted carefully to pH 3.6 and filtered quickly through cotton, its activity was tested on the same type of substrate; namely, milk of pH 5. At 26.6° there was complete activation of the prorennin in 7 minutes (see Table I). Propepsin, on the other hand, is only about one-third activated under the same conditions. We find the difference to be even more marked if milk of pH 6.3, as previously employed by us, is used. The destruction of pepsin we reduced to a minimum by working with concentrated pepsin solutions. It is to be noted that there is not a sharp line between the inactive and the completely activated states; *i.e.*, partial activation is possible if the pH is not quite at the most favorable point.

As regards the activation of prorennin by bacteria, which Ege and Lundsteen describe, we may say that we have also noticed that aqueous solutions of prorennin become active if not preserved, owing to bacterial action. It is possible, as they maintain, that this effect is due to the proteolytic enzymes of the bacteria. In this connection we wish to point out that the activation of prorennin which Ege and Lundsteen brought about by the use of pancreatin may have been an apparent activation and not a real one; that is, the clotting may have been caused by the pancreatin itself. Certain dilute concentrations of trypsin clot milk whereas more concentrated ones do not (6).

TABLE I

Showing Difference in Activation of Propepsin and Prorennin at Various pH Values

The milk was of pH 5 and buffered (3). The results are expressed as cc. of milk clotted in 10 minutes by 1 cc. of enzyme solution at 37°.

pH	Propepsin	pH	Prorennin
7.1*	1:0.4†	7.2*	1:1,900†
3.6	1:5,400	3.6	1:10,000
1.6	1:18,000	1.6	1:10,000

* Original extract.

† Preformed activity.

Experiments now in progress support Hedin's (7) contention that the gastric proteases differ with every species of animal. We find this to be true of the pH values at which their precursors are activated as well as of their other properties.

In a personal communication Professor Sørensen has called our attention to two statements of ours which are contradictory to each other. In a recent paper (2) we stated that propepsin differs from prorennin in that the former cannot clot milk whereas the latter can, while in an earlier paper (1) we had stated that prorennin could be obtained free of rennet activity. This earlier statement was correct and the later one was, unfortunately, carelessly phrased. We intended to say that the neutral extracts containing prorennin or propepsin show the difference stated, owing to the presence of preformed rennin in the extract of the fourth stomach of the calf. Neither one of the zymogens possesses actual clotting power.[1]

SUMMARY

Prorennin and propepsin (pepsinogen) become activated at distinctly different pH values; the former becomes completely active at pH 3.6 and the latter at pH 1.6 under given conditions. Partial activation of either may occur at lower hydrogen ion concentrations.

BIBLIOGRAPHY

1. Kleiner, I. S., and Tauber, H., *J. Biol. Chem.*, **96**, 755 (1932).
2. Tauber, H., and Kleiner, I. S., *Z. physiol. Chem.*, **220**, 205 (1933).
3. Ege, R., and Lundsteen, E., *Biochem. Z.*, **268**, 164 (1934).
4. Ege, R., and Thygesen, P. M., *Biochem. Z.*, **264**, 3 (1933).
5. Truog, E., *Science*, **74**, 633 (1931).
6. Tauber, H., and Kleiner, I. S., *J. Biol. Chem.*, **104**, 271 (1934).
7. Hedin, S. G., *Z. physiol. Chem.*, **74**, 242 (1911).

---

[1] We are also indebted to Professor Sørensen for calling our attention to a typographical error on page 756, Experiment 4 (1); *0.04* N *HCl* should read *0.4* N *HCl*.

A MANOMETRIC MICROMETHOD FOR ARGINASE DETERMINATION

ENZYMATIC STUDY OF BLOOD ARGINASE IN RATS

By LEOPOLD WEIL and MARY A. RUSSELL

(*From the Cancer Research Laboratories, University of Pennsylvania Graduate School of Medicine, Philadelphia*)

(Received for publication, June 14, 1934)

In a recent communication Waldschmidt-Leitz, McDonald, and coworkers (1) presented a detailed report of the enzymatic equipment of transplantable rat tumor tissue. On the basis of their data, arginase assumes a conspicuous position; the increase found in tumor arginase activity as the tumor ages may well be a characteristic quality of pathologic, rapidly growing tissue. The results of Edlbacher and Merz (2) and of Klein and Ziese (3) likewise indicate the importance of this enzyme in tumor metabolism. Weil (4) has shown that during the aging of the tumor the increase in its arginase activity is accompanied by a progressive decrease in liver arginase. In view of this relation, a study of blood arginase activity became of interest.

Since the concentration of arginase in blood is very small, a new method has been developed which permits the measurement of much smaller amounts of this enzyme than is possible by the usual procedure of Edlbacher and Röthler (5). The enzyme is allowed to decompose arginine into ornithine and urea in the usual manner. The urea formed is then treated with urease, the CO_2 produced being measured manometrically in the Warburg apparatus. The NH_3 simultaneously formed remains in solution at the acid reaction employed.

By this method, rat blood arginase was found to be dependent upon the sexual condition of the animals. A similar relationship has been established by Edlbacher and Röthler (6) between sex and liver arginase concentration. Rat blood arginase appeared to be fully activated, since addition of activator (7) (cysteine +

Fe″) produced no increase in activity, such as is found in human and horse blood.

To study the influence of sexual development upon blood arginase in the case of male rats, we investigated young animals (50 to 60 gm.), and obtained an average value, expressed in microarginase units, of 1.25 in 1 cc. of blood. This value shows a tendency to increase with maturity to an average of 3.11 units per cc. of blood. After castration of mature rats the blood arginase concentration decreased; in 1 month after castration the average value found was 1.07 units per cc. of blood.

Normal mature female rats showed, in comparison with male rats, a relatively low arginase concentration, the average found being 1.6 units per cc. of blood. Pregnancy and spaying did not appear to have any influence on the blood arginase.

As previously mentioned, arginase has an important part in tumor metabolism. Investigations in this direction indicated that mature male rats with transplantable cancer have a slight tendency to possess a low blood arginase content. The average value obtained was 1.91 units per cc. of blood. This decrease may be related to the diminished arginase activity of the liver in rats with cancer (4).

With reference to the function of arginase, the important work of Krebs and Henseleit (8) has indicated a connection between the enzyme and urea formation. Therefore, the relation between arginase activity and blood urea content was studied. No obvious relationship was found, however. Nevertheless, this connection may exist, since arginine injected subcutaneously increases the blood urea concentration considerably without stimulating the blood arginase activity. It is possible that the arginine is decomposed before entering the blood stream, and that the latter merely takes up the decomposition products. This would explain why injection of arginine increases the blood urea without increasing the blood arginase concentration in the sense of an Abderhalden reaction.

EXPERIMENTAL

Description of Method—In the former method for arginase determination, developed by Edlbacher and Röthler (5), the urea formed from arginine under the influence of arginase is decomposed by urease, and the ammonia liberated is then distilled and titrated with 0.02 N H_2SO_4. The limit of accuracy corresponds to about 0.6 mg. of urea. The new method is similar in principle, except that the CO_2 formed from the urea by the action of urease is measured manometrically in the Warburg apparatus. Krebs and Henseleit (8) have used a similar method for the direct determination of urea. Since twice as much ammonia is formed as CO_2, it might appear desirable to measure the ammonia formation manometrically. However, ammonia has such a high absorption coefficient that even at pH 10 or 11, with paraffin oil as manometer fluid, no ammonia evolution can be detected. The reaction with urease is carried out at pH 5, and at this acid reaction the ammonia is effectively bound. The limit of accuracy of this method corresponds to about 0.003 mg. of urea; the sensitivity is thus 200 times that of the distillation method.

So far, the new method has been applied almost exclusively to blood. The quantity of blood required for one determination depends on the animal used. Rat blood requires 1 cc., while horse blood and human blood require only 0.1 to 0.2 cc.[1] In all cases oxalated blood was used, since it was found that the oxalate does not interfere with the determination. With rats, the blood was removed from the heart under ether anesthesia. Small amounts of urease were found to be present in blood and tissues, but at pH 9.5 they cause no interference.

After removal from the animal, the blood is adjusted to the optimum pH (9.5) for arginase activity by means of 0.1 N NaOH, the pH being controlled by potentiometric measurement with the hydrogen electrode. Adjusted in this manner, the pH shows no significant shift during the determination. Glycocoll-NaOH buffer was not used because it was found to have a slight inhibiting effect. Usually 0.45 to 0.5 cc. of 0.1 N NaOH is sufficient to adjust the pH of 1 cc. of blood to 9.5.

The desired amount of this blood (pH 9.5) is accurately measured into a manometer vessel and 0.5 cc. of arginine-HCl (containing 4 mg., and previously adjusted to pH 9.5 with NaOH) is added. Sufficient water is added to bring the volume up to 3.2

---

[1] The blood arginase activity in the case of human blood is confined to the cells; plasma has no inhibiting effect on the enzyme activity. In the case of rat blood the arginase is found in both the cells and in the plasma.

cc. A drop of toluene is added to prevent bacterial infection. The mixture is incubated at 30° for 20 hours. 0.5 cc. of 3 N acetate buffer of pH 5 is then added, and 0.3 cc. of urease solution[2] is placed in the side arm of the manometer vessel. The latter is attached to the manometer and shaken at 30° for 30 to 40 minutes, to expel the blood CO_2. When no further pressure change occurs, the urease solution is tipped into the main chamber of the reaction vessel, and the manometer shaken until all the urea has been decomposed (30 to 40 minutes). Since blood contains considerable amounts of urea, it is always necessary to run a control. This is carried out in exactly the same way as the regular determination, except that the arginine-HCl is not added until immediately before the urea determination is made. The difference between the two determinations expresses the arginase influence.

For converting the manometer reading (pressure in mm. of Brodie fluid) into c.mm. of CO_2 formed, it is necessary to determine the vessel constant. A known quantity of urea is decomposed by urease, and the CO_2 formed measured. The theoretical amount of CO_2 (in c.mm.) divided by the observed pressure change (in mm. of Brodie fluid) gives the vessel constant. In any given determination, the observed pressure change (in mm.) multiplied by the vessel constant gives the CO_2 formation in c.mm. at 30°. For example, to 0.5 cc. of urea solution (0.5 mg. of urea) were added 2.7 cc. of water and 0.5 cc. of 3 N acetate buffer of pH 5.0. The urea determination was carried out exactly as previously described, 0.3 cc. of urease solution being tipped in from the side arm of the manometer vessel. The observed pressure difference at the end of the reaction was 138 mm. of Brodie fluid; the theoretical CO_2 formation was 205.7 c.mm. at 30°; the quotient, 1.490, represents the vessel constant at 30°. Urea determinations by this method agreed with the results obtained on similar samples by distillation of the ammonia formed.

Rate of Reaction of Blood Arginase—For the quantitative measurement of blood arginase, it was necessary to determine the time course of the reaction. For this purpose horse blood was taken, since the arginase concentration in this is much higher than in rat blood. For each determination, 1 cc. of blood was taken. Since horse blood arginase requires activation, it was allowed to stand for 1 hour at pH 7.0 and 30° with 0.2 cc. of cysteine solution (2 mg. of cysteine-HCl at pH 7.0) and 0.1 cc. of 0.1 N $FeSO_4$ solution. After this preliminary activation period, the pH was adjusted to 9.5 with NaOH, and the substrate added (0.5 cc., containing 4 mg. of arginine-HCl at pH 9.5). At various time intervals, the urea formation was measured as previously described. A blank was run simultaneously. Table I shows the results obtained in c.mm. of CO_2 at 30°.

---

[2] 6 gm. of Arlco jack bean meal are allowed to stand overnight with 20 cc. of water and 1 cc. of 1 N acetic acid. After centrifuging, the supernatant liquid is used.

Relation between Quantity of Blood Arginase and Activity—Since the rate of reaction of blood arginase decreases with time (Table I), it became necessary to establish the relation between quantity of enzyme and activity, or degree of arginine splitting. This relationship permitted us to set up a blood arginase unit for quantitative comparisons of enzyme content. For this purpose 10 cc. of horse blood were fully activated by allowing them to stand for 1 hour at 30° with 2 cc. of cysteine solution (containing 20 mg. of cysteine-HCl at pH 7.0), and 0.1 cc. of 0.1 N $FeSO_4$. The blood was then adjusted to pH 9.5 with NaOH as previously described. Increasing amounts of this blood were then treated with the usual amount of arginine-HCl (pH 9.5) and water, and incubated for 20 hours at 30°. At the end of the incubation the urea formed was determined in the usual manner. For each determination a control was run without substrate. The results are expressed in Table II in c.mm. of CO_2 formed at 30°.

TABLE I

Reaction Rate of Blood Arginase

Time, *hrs.*	2	4	6	8	9	10
Arginase activity, *c.mm. CO_2*	52	100	135	160	174	195

TABLE II

Relation between Quantity of Blood Arginase and Activity

Blood, *cc.*	0.05	0.01	0.2	0.3	0.4	0.5
Arginase activity, *c.mm. CO_2 at 30°*	63	122	190	233	256	271

The unit for blood arginase was taken as that quantity of enzyme which when incubated, for 20 hours at 30° and pH 9.5, with 4 mg. of arginine-HCl (adjusted to pH 9.5) in a total volume of 3.2 cc., will produce an amount of urea equivalent to 20 c.mm. of CO_2 at 30°, the latter being determined as previously described. Fig. 1 shows that the relation between enzyme units and CO_2 formation is linear up to 6 units.

Blood Arginase in Male Rats at Various Sexual Stages—In this series, the blood arginase of young male rats (50 to 60 gm.), of mature male rats (averaging 250 gm.), and of mature male rats 1 to 1.5 months after castration was investigated. For each determination 1 cc. of oxalated blood was taken and analyzed as previously described. Table III shows the results obtained in arginase units per cc. of blood.

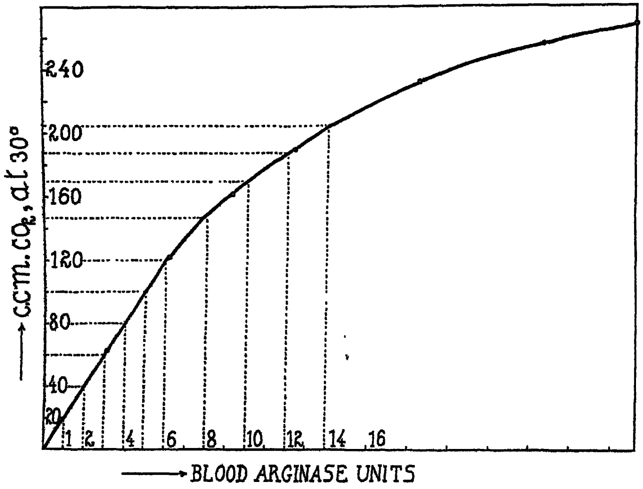


Fig. 1. Relation between quantity of enzyme and activity

Simultaneously, the urea content of the blood was measured. To 1 cc. of blood were added 0.5 cc. of 3 N acetate buffer (pH 5.0) and 2.2 cc. of water. After the blood CO_2 was expelled by shaking in the Warburg apparatus, 0.3 cc. of urease solution was tipped into the main chamber of the manometer vessel. The CO_2 formation was measured at 30° and converted into mg. of urea.

TABLE III

Blood Arginase and Urea in Male Rats at Various Sexual Stages

Young rats		Mature rats		Castrated rats	
Arginase	Urea	Arginase	Urea	Arginase	Urea
units per cc.	*mg. per cc.*	*units per cc.*	*mg. per cc.*	*units per cc.*	*mg. per cc.*
2.05	0.248	5.4	0.270	1.1	0.146
2.7	0.317	3.4	0.273	0.9	0.234
5.5	0.243	3.8	0.292	1.2	0.295
1.05	0.228	4.6	0.168	1.15	0.204
0.05	0.307	5.3	0.258	1.05	
0.8	0.241	3.3	0.351	0.0	0.265
0.2	0.326	3.1	0.309	1.45	0.282
0.0	0.360	1.1	0.390	1.8	0.343
0.0	0.307	1.2	0.258	1.0	
1.0		1.1	0.417		
0.5		2.3	0.309		
		2.6	0.329		
		3.05			
Average...1.25		3.11		1.07	

TABLE IV

Blood Arginase and Urea in Rats with Cancer

Arginase	Urea
units per cc.	*mg. per cc.*
4.5	0.300
4.5	0.331
2.9	0.185
2.2	0.314
0.5	0.382
1.8	0.365
1.2	0.380
0.4	0.331
2.2	0.354
2.1	0.259
0.7	0.282
1.8	0.343
0.1	0.364
Average......1.91	

Blood Arginase in Rats with Transplantable Cancer—Mature male rats were inoculated with Philadelphia No. 1 sarcoma, and after 3 to 6 weeks the blood was examined for arginase as previously described. Table IV shows the arginase content in units per cc., and the urea in mg. per cc.

TABLE V

Blood Arginase and Urea in Female Rats at Various Sexual Stages

Normal		Pregnant		Spayed	
Arginase	Urea	Arginase	Urea	Arginase	Urea
units per cc.	*mg. per cc.*	*units per cc.*	*mg. per cc.*	*units per cc.*	*mg. per cc.*
3.05	0.302	1.1	0.290	1.95	
3.0	0.256	4.4	0.207	2.7	0.307
0.45	0.182	4.45		0.95	
1.15	0.343	2.3		0.3	0.219
2.5	0.265	1.05	0.259	0.5	0.283
0.4	0.317	0.8	0.382	1.0	0.300
1.3	0.331	1.6	0.370	2.5	
2.1	0.368	1.7			
0.6	0.324				
2.65	0.268				
0.0	0.263				
2.2	0.226				
Average...1.61		2.25		1.31	

TABLE VI

Effect of Subcutaneous Injection of Arginine on Blood Arginase and Urea

Arginase	Urea
units per cc.	*mg. per cc.*
1.9	0.559
0.0	0.537
1.4	0.502
1.7	0.512

Blood Arginase in Female Rats at Various Sexual Stages—In this series the blood arginase of mature female rats, pregnant rats, and mature spayed female rats was investigated. In the case of the spayed animals, the blood was analyzed 1 to 1.5 months after removal of the ovaries. The results are shown in Table V.

Blood Arginase and Urea after Subcutaneous Injection of Arginine—Four male rats (130 gm.) were injected subcutaneously at 8 hour intervals with 0.3 gm. of arginine-HCl (neutralized to pH 7.0) in 2 cc. of water. About 5 hours after the third injection, the blood arginase and blood urea were determined by the usual methods. The results are shown in Table VI.

The authors are indebted to Doctor Ellice McDonald, Director, for advice and assistance.

SUMMARY

A manometric micromethod for arginase determination is described. A unit has been defined for quantitative comparisons of arginase activity.

Mature male rats show a tendency to possess a higher blood arginase content than young or castrated male rats. Male rats with transplantable cancer also have a tendency toward low blood arginase.

Female rats have a relatively lower blood arginase content than male rats. Neither pregnancy nor spaying has any noticeable effect on blood arginase.

No parallelism was found between blood arginase and blood urea content.

BIBLIOGRAPHY

1. Waldschmidt-Leitz, E., McDonald, E., *et al.*, *Z. physiol. Chem.*, **219**, 115 (1933).
2. Edlbacher, S., and Merz, K. W., *Z. physiol. Chem.*, **171**, 251 (1927).
3. Klein, G., and Ziese, W., *Z. Krebsforsch.*, **37**, 323 (1932).
4. Weil, L., unpublished data.
5. Edlbacher, S., and Röthler, H., *Z. physiol. Chem.*, **148**, 264 (1925).
6. Edlbacher, S., and Röthler, H., *Z. physiol. Chem.*, **148**, 273 (1925).
7. Purr, A., and Weil, L., *Biochem. J.*, **28**, 740 (1934).
8. Krebs, A. H., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).

STUDIES ON OXIDATION-REDUCTION

XXI. PHTHIOCOL, THE PIGMENT OF THE HUMAN TUBERCLE BACILLUS

By ERIC G. BALL

(*From the Department of Physiological Chemistry, the Johns Hopkins University, School of Medicine, Baltimore*)

(Received for publication, June 21, 1934)

INTRODUCTION

More data are needed on those constituents of living tissue which are components of definite oxidation-reduction systems. Such information will aid in defining the intensity level of energy changes in living cells; a level essential for the activation of certain enzymes (22) and for the protection from destruction of compounds like epinephrine (5). Phthiocol, a pigment isolated from the tubercle bacillus by Anderson and Newman (2), has been shown by these workers (3, 4) to be 2-methyl-3-hydroxy-1,4-naphthoquinone. On the basis of its structure this compound should be amenable to potentiometric study. Data will be presented in this paper to show that phthiocol is the oxidant of a reversible system whose potential in the more usual pH range is among the lowest reported for systems of biological origin.

Results

The phthiocol used in this investigation was a synthetic sample kindly supplied by Professor R. J. Anderson. This compound is a weak acid, poorly soluble in aqueous solutions of pH lower than its pK value (5.08), more readily soluble on the alkaline side. In aqueous solutions the undissociated form has a pale yellow color, the anion a dark red. The reduced form of each species is colorless.

Since both components of the system were entirely stable throughout the usual pH range, the procedure for potentiometric measurement was similar to that described in earlier papers of this series. Veronal buffers as described by Michaelis (24) were substituted for the borate system since borates cannot be used with polyhydroxy compounds. Corrections were made for change of potential attributable to those changes of pH which are caused by the products formed in the oxidation-reduction process. All potentials here recorded are at 30° and have been brought to the hydrogen standard in accordance with the conventions of Clark (10).

TABLE I

Titration of Reduced Phthiocol

Phthiocol, 0.0005 M, in phosphate buffer (0.1 M); pH 7.321; reduced with H_2 and platinum black; 50 ml. titrated with $K_3Fe(CN)_6$, 0.005 N; dissolved in the same buffer solution. Temperature 30.0°.

Oxidation	0.03006 log $[S_r]/[S_o]$	E_h observed	E_h corrected*	E'_0	Deviation from average
per cent					
3.90	+0.04183	−0.24975	−0.24986	−0.20803	+0.00016
6.47	+0.03487	−0.24285	−0.24302	−0.20815	+0.00004
9.07	+0.03009	−0.23809	−0.23833	−0.20824	−0.00005
11.66	+0.02643	−0.23439	−0.23470	−0.20827	−0.00008
16.77	+0.02092	−0.22866	−0.22911	−0.20819	0.00000
21.90	+0.01660	−0.22422	−0.22481	−0.20821	−0.00002
32.27	+0.00968	−0.21705	−0.21792	−0.20824	−0.00005
42.53	+0.00393	−0.21101	−0.21216	−0.20823	−0.00004
52.87	−0.00150	−0.20528	−0.20671	−0.20821	−0.00002
63.27	−0.00710	−0.19941	−0.20112	−0.20822	−0.00003
73.65	−0.01342	−0.19280	−0.19479	−0.20821	−0.00002
83.88	−0.02153	−0.18440	−0.18666	−0.20819	0.00000
94.28	−0.03658	−0.16910	−0.17164	−0.20822	−0.00003
96.84	−0.04468	−0.16070	−0.16331	−0.20799	+0.00020
Average................................				−0.20819	

* Correction for acid formed on oxidation, total change 2.7 millivolts.

Table I contains the results of a typical titration. Blank platinum or gold-plated platinum electrodes registered the same potentials except at the extremes of the curve, where the gold-plated electrode was accepted as furnishing the most reliable potential. The uniformity of the *E'*$_0$ values over the whole range of oxidation furnishes proof that 2 electrons are involved in the reaction.

The value of $n = 2$ was found to hold also at other pH values and no indication of a two-step oxidation was encountered as described for the bacterial pigments, pyocyanine (Friedheim and Michaelis (20), Elema (14)) and chlororaphine (Elema (15)).

The relation of E'_0 to pH is given in Table II. Inspection of Table II shows that the potentials are independent of the titrating agent employed and of moderate changes in the concentration of phthiocol. In those experiments in which an oxidizing agent was employed in the titration, the pigment was previously reduced with hydrogen in the presence of platinum black. Reoxidation will give rise to the original quinone since Fieser (16, 17) has shown that a solution of hydroxynaphthoquinone consists of a mixture

TABLE II

Relation of E'_0 to pH

Temperature 30.0°.

Titrating agent	Buffer	Phthiocol concentration	pH	E'_0
		M		
Quinone	HCl	0.0001	1.120	+0.2314
Mixture	Phosphate	0.0001	2.145	+0.1699
"	Citrate	0.0001	2.948	+0.1215
"	Acetate	0.0001	4.119	+0.0497
"	"	0.0001	4.643	+0.0156
Ferricyanide	"	0.0001	4.653	+0.0165
Mixture	"	0.0001	5.241	−0.0283
"	Phosphate	0.00005	5.841	−0.0770
"	"	0.0001	6.500	−0.1350
$Na_2S_2O_4$	"	0.0005	7.321	−0.2059
Ferricyanide	"	0.0005	7.321	−0.2082
Iodine	"	0.0005	7.321	−0.2079
Ferricyanide	"	0.0001	7.344	−0.2100
Mixture	"	0.0001	7.344	−0.2101
"	Veronal	0.0001	8.437	−0.3067
"	"	0.0001	9.065	−0.3558
Ferricyanide	Phosphate	0.0001	10.500	−0.4456
Mixture	"	0.0001	10.870	−0.4645
"	"	0.0001	11.366	−0.4909
"	NaOH + KCl	0.0001	11.546	−0.5034
"	" + "	0.0001	12.260	−0.5301
"	" + "	0.0001	12.561	−0.5396

of tautomers with the orthoquinone form present in extremely small concentration. Data given under the heading of "Mixture" were obtained with a mixture of oxidant and reductant produced by partial air oxidation of reduced phthiocol. The percentage oxidation of the mixture was determined by its potential in a buffer solution previously used in a titration experiment.

The complete electrode equation stated with numerical coefficient for 30° is as follows:

$$E_h = E_0 + 0.03006 \log \frac{[S_o]}{[S_r]} + 0.03006 \log \frac{[K_{r_1} K_{r_2} K_{r_3} + K_{r_1} K_{r_2} (\mathrm{H}^+) + K_{r_1} (\mathrm{H}^+)^2 + (\mathrm{H}^+)^3]}{K_o + (\mathrm{H}^+)}$$

Here (H^+) is the hydrion activity, $[S_o]$ and $[S_r]$ the molar concentrations of total oxidant and total reductant. For 30°, the normal potential, E_0, is 0.2987; the apparent dissociation constant of the hydroxyl group of the oxidant, K_o, is 8.32×10^{-6} and the first and second apparent dissociation constants of the reductant, K_{r_1} and K_{r_2} are 1.26×10^{-9} and 2.88×10^{-12}. The dissociation of the third hydroxyl group of the reductant was not effective in the pH range studied, so that the constant K_{r_3} may be considered negligibly small and hence the term $K_{r_1} K_{r_2} K_{r_3}$ omitted from the equation.

In Fig. 1 the curve labeled phthiocol has been drawn to fit values calculated by the equation given above. The experimentally determined points are appropriately marked.

DISCUSSION

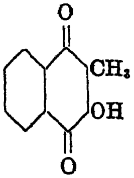
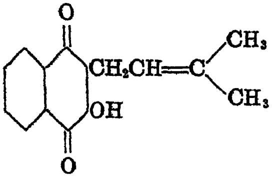
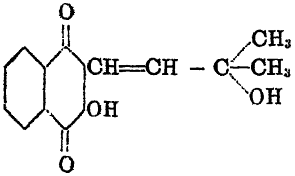
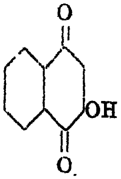
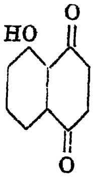
Hydroxynaphthoquinones possessing the grouping $\mathrm{O{=}\overset{|}{C}{-}CH}$ $\mathrm{{=}\overset{|}{C}{-}OH}$, which Claisen (9) first observed is almost equivalent to a carboxyl group, are known to exhibit the properties of weak acids. It is therefore not surprising to find that the hydroxyl group in phthiocol possesses a pK value of 5.08. Reduction to the corresponding naphthohydroquinone causes a marked change in the acid properties of the compound. The first dissociable hydrion of the reductant has a pK value of 8.90. As a result the system exhibits a value of 0.09 for $-\frac{\Delta E'_0}{\Delta \mathrm{pH}}$ in the intermediate pH range.

Consequently the system reaches more negative values in the alkaline region than would be expected from the values reported by Fieser (17) for similar compounds in acid solutions.

It is of interest to know whether the oxidant or the reductant of this system predominates in the living tubercle bacillus. Though Anderson and Newman (2) isolated the oxidant, the possibility exists that air oxidation of the reductant, which takes place readily, may have occurred during isolation. Dr. Anderson informs me that investigations are under way which may settle this point. The fact that colonies of the organism are described as possessing a yellowish tint is suggestive that it is the oxidized form which is present. The occurrence of the reductant of such a negative oxidation-reduction system in an aerobic organism would not be in agreement with previous data concerning the intensity level of oxidation-reduction processes in living aerobic tissue. Cultures of tubercle bacillus according to Aksianzew (1) register potentials ranging from +0.300 volt to +0.005 volt. If such potentials are an indication of conditions within the cell the phthiocol system would be maintained completely in the oxidized state.

Hydroxynaphthoquinones isolated from natural sources, together with the normal potential of their systems, are listed in Table III. All except phthiocol are of plant origin. Lapachol and lomatiol resemble phthiocol most closely in structure and consequently their systems exhibit normal potentials of the same magnitude. The system of which lawsone is the oxidant has a normal potential about 50 millivolts positive to that of the phthiocol system. The substitution of a methyl group in the (2) position tends not only to lower the potential of the system but also to decrease about 10-fold the dissociation constant of the hydroxyl group of the oxidant (see Fig. 1). In the interpretation of the E'_0-pH curve of lawsone, Friedheim (19) has neglected the fundamental principles involved in the use of values for $-\frac{d^2E'_0}{d\text{pH}^2}$. In Fig. 1 the curve has been drawn through Friedheim's experimental points in such a manner as to indicate a dissociation exponent of the oxidant at 3.85 and exponents of the reductant at 9.1 and 11.1. The juglone system is the most positive of the group. On the basis of structural differences this is to be expected since the sub-

Hydroxynaphthoquinones from Natural Sources

Compound	Source	E'₀ of system		
			Temperature	Reference
		volt	°C.	
 Phthiocol	As oxidant (?) in human tubercle bacillus (2)	0.2987	30	
 Lapachol	As oxidant (?) in a variety of woods (see Fieser (18))	0.287*	25	(17)
 Lomatiol	As oxidant (27) in seeds of *Lomatia ilicifolia*	0.294*	25	(17)
 Lawsone	As oxidant (19) in henna plant	0.352 0.356*	20 25	(19) (12)
 Juglone	As reductant (26) in walnut shells	0.435 0.452*	20 25	(19) (12)

* Solvent: 50 per cent alcohol solution, 0.5 N or 0.1 N in HCl.

stitution of hydroxyl or alkyl groups is well known to effect a more pronounced change in potential of the system if made in the same ring that contains the quinone grouping. The occurrence of the reduced form of juglone in living cells is in keeping with the higher potential of this system.

Three other naturally occurring hydroxynaphthoquinones have been described. Plumbagin, isolated first from the roots and stems of *Plumbago europæa* by Madinaveitia and Gallego (23), is thought to be a methyl juglone; the position of the methyl radical is unknown. Rennie (28) has isolated from the reddish sap of the tubers of *Drosera whittakeri* two pigments. He believes they are trihydroxymethyl- and dihydroxymethylnaphthoquinones. The position of the substituted groups is unknown. Both of these compounds should be of interest for a potentiometric study, for if they possess such structures it is possible they are the oxidants of systems considerably more negative than phthiocol.

The relation of the phthiocol system to a number of other naturally occurring systems is shown in Fig. 1. A consideration of the three systems, the oxidants of which are a lyochrome from mammalian tissue (7), echinochrome (8), and phthiocol, shows that each in turn, as the pH increases, becomes the lowest of the known systems of biological origin. Of particular interest is the relation to the lactate-pyruvate system. The values given by Barron and Hastings (6) for this system coincide at pH 7.0 with the phthiocol system. Phthiocol therefore should be a valuable mediator for the study of this electromotively inactive system in the biological pH range. Such a study is planned. Conant and Pappenheimer's (13) value for the methemoglobin system, and Green's (21) data for the cytochrome C system were used in Fig. 1. The source of the data for other systems shown has been cited earlier in the text.

Nearly a dozen pigments from natural sources have been shown to form reversible oxidation-reduction systems. All of these are not shown in Fig. 1 but lie clustered between the juglone and echinochrome systems at pH values 7.0 to 8.0. Most of these systems lie between those regions of potential shown by Cohen *et al.* (11) to represent the state of reducing intensity of ameba in aerobic and anaerobic conditions (see Fig. 1). This interesting fact, coupled with the rapidity with which the reductants of these systems are oxidized by air, has led to the assignment of the rôle of accessory respiratory ferments to these pigments. Such indeed may be their rôle though the increased oxygen consumption caused by their addition to tissue to which they are foreign is not a convincing test of their physiological importance in normal respira-

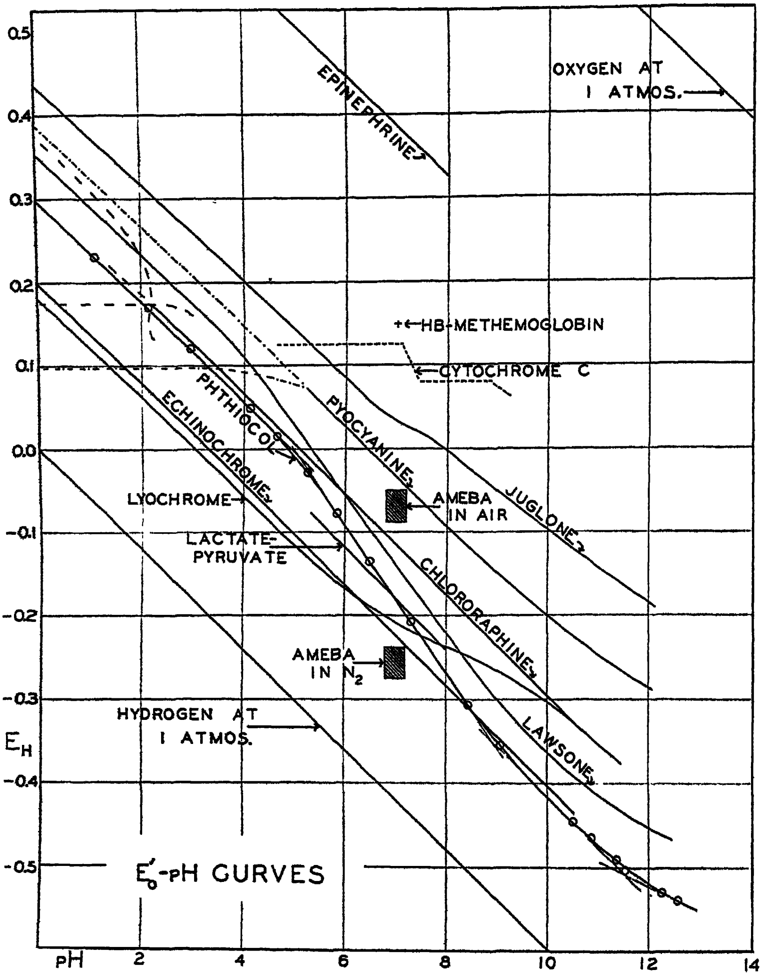


FIG. 1

tion; especially since increases in quantity of oxygen consumed give no indication as to the usefulness of the energy released to the normal processes of the cell. Most of these compounds occur in lower organisms or plants in which the chief method of respiration is still unknown so that the assignment of even a minor rôle in this process, even in their native tissue, seems at present premature.

Phthiocol should form a valuable addition to the series of oxidation-reduction indicators at pH values greater than 6.0. Its solubility and color intensity in this range are satisfactory. At pH 7.4 the system lies intermediate to the indigo-monosulfonate (30) and phenosafranine (29) systems and slightly negative to the less desirable brilliant alizarin blue system (25) which heretofore was the only one available in this region.

SUMMARY

Phthiocol, 2-methyl-3-hydroxy-1,4-naphthoquinone, a pigment of the human tubercle bacillus is the oxidant of a reversible oxidation-reduction system whose potential is among the lowest reported for systems of biological origin. Values for the E'_0 of this system at pH values ranging from 1.1 to 12.6 are reported; the normal potential at 30° is 0.2987 volt. The following dissociation constants are assigned: for the oxidant $K_o = 8.32 \times 10^{-6}$, for the reductant $K_{r_1} = 1.26 \times 10^{-9}$ and $K_{r_2} = 2.88 \times 10^{-12}$. The relation of this system to other naturally occurring systems, especially those whose oxidants are also hydroxynaphthoquinones, is discussed. Phthiocol is suitable for use as an oxidation-reduction indicator at pH values more alkaline than 6.0. In this range the potential of the system lies in a region heretofore sparsely covered by indicators.

BIBLIOGRAPHY

1. Aksianzew, M. I., *Z. Tuberk.*, **68**, 249 (1933).
2. Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, **101**, 773 (1933).
3. Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, **103**, 197 (1933).
4. Anderson, R. J., and Newman, M. S., *J. Biol. Chem.*, **103**, 405 (1933).
5. Ball, E. G., and Chen, T.-T., *J. Biol. Chem.*, **102**, 691 (1933).
6. Barron, E. S. G., and Hastings, A. B., *J. Biol. Chem.*, **100**, xi (1933).
7. Bierich, R., and Lang, A., *Z. physiol. Chem.*, **223**, 180 (1934).
8. Cannan, R. K., *Biochem. J.*, **21**, 184 (1927).
9. Claisen, L., *Ann. Chem.*, **291**, 35 (1896).
10. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 461–482 (1928).

11. Cohen, B.; Cold Spring Harbor symposia on quantitative biolog New Bedford, **1**, 214 (1933). Cohen, B., and Chen, T.-T., *Proc. So Exp. Biol. and Med.*, **31**, 115 (1933).
12. Conant, J. B., and Fieser, L. F., *J. Am. Chem. Soc.*, **46**, 1858 (1924).
13. Conant, J. B., and Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **98**, (1932).
14. Elema, B., *Rec. trav. chim. Pays-Bas*, **50**, 807 (1931).
15. Elema, B., *Rec. trav. chim. Pays-Bas*, **52**, 569 (1933).
16. Fieser, L. F., *J. Am. Chem. Soc.*, **48**, 2922 (1926).
17. Fieser, L. F., *J. Am. Chem. Soc.*, **50**, 439 (1928).
18. Fieser, L. F., *J. Am. Chem. Soc.*, **49**, 857 (1927).
19. Friedheim, E. A. H., *Biochem. J.*, **28**, 180 (1934).
20. Friedheim, E., and Michaelis, L., *J. Biol. Chem.*, **91**, 355 (1931).
21. Green, D. E., *Proc. Roy. Soc. London, Series B*, **114**, 423 (1934).
22. Hellerman, L., Perkins, M. E., and Clark, W. M., *Proc. Nat. Acad. Sc* **19**, 855 (1933).
23. Madinaveitia, A., and Gallego, M., *An. Soc. españ. fisic. y quim.*, **2** 263 (1928). Tummin Katti, M. C., and Patwardhan, V. N., *J. India Inst. Sc.*, **15 A**, 9 (1932).
24. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
25. Michaelis, L., and Eagle, H., *J. Biol. Chem.*, **87**, 713 (1930).
26. Mylius, F., *Ber. chem. Ges.*, **17**, 2411 (1884).
27. Rennie, E. H., *J. Chem. Soc.*, **51**, 371 (1887).
28. Rennie, E. H., *J. Chem. Soc.*, **63**, 1083 (1893).
29. Stiehler, R. D., Chen, T.-T., and Clark, W. M., *J. Am. Chem. Soc* **55**, 891 (1933).
30. Sullivan, M. X., Cohen, B., and Clark, W. M., *Pub. Health Rep., U. P. H. S.*, **38**, 1669 (1923).

THE VITAMIN C CONTENT OF HUMAN TISSUES*

By MARTIN YAVORSKY, PHILLIP ALMADEN, and C. G. KING

(*From the Department of Chemistry, University of Pittsburgh, Pittsburgh*)

(Received for publication, June 16, 1934)

The development of a direct chemical method for the estimation of vitamin C in small amounts of tissue has made possible a new approach to the study of latent human scurvy and related physiological problems. Although absolute specificity for the vitamin cannot be claimed for the reaction used in its determination (1), for most tissues the chemical method gives in a few minutes time a more precise measure of the vitamin content of a single bit of tissue than could be obtained by feeding large amounts of such material over a long period of time. Furthermore, the feeding technique cannot be applied to the study of small and essentially restricted amounts of tissue such as are included in the present investigation. It would also meet with a certain degree of esthetic objection.

It is clear from the data which follow (*a*) that the distribution of vitamin C in human tissues is analogous to that found in guinea pigs (1), (*b*) that the concentration varies over a wide range with different individuals, and (*c*) that latent human scurvy, generally unobserved in clinical practice, is fairly common.

The rapid elimination of large quantities of vitamin C after ingestion (and the continued loss from the body without a dietary source being supplied) has been shown by Harris, Ray, and Ward (2). Dalldorf, using the capillary resistance test (3), and Hanke, in studying dental disorders especially (4), have recently presented evidence of wide-spread latent scurvy. The lesions which result from vitamin C deficiency (5) give indications of an intimate relation to general tissue functions.

---

* Contribution No. 284 from the Department of Chemistry, University of Pittsburgh.

EXPERIMENTAL

The method used for determining vitamin C was essentially that described previously (1), in which a solution of 2, 6-dichlorophenolindophenol (approximately 0.003 M) is added directly to a trichloroacetic acid (8 to 10 per cent) extract of the finely ground tissue. The dye is reduced rapidly by the vitamin, giving an end-point for the titration within 5 to 10 seconds, thus avoiding in large degree at least the interference of slower reducing substances such as glutathione.

The tissues used for analysis were obtained from hospital autopsies and used within 24 hours after death. During this interval, as shown by repeated tests on analogous tissues, there would not be a serious loss (less than 10 per cent) in titration value.

TABLE I

Vitamin C Content of Tissues in Different Age Groups (Mg. per Gm.)

Age group No. of cases	1–30 days 11	1–12 mos. 9	1–10 yrs. 11	11–45 yrs. 17	46–77 yrs. 19
Adrenal	0.581	0.525	0.550	0.393	0.230
Brain	0.460*	0.189*	0.433*		0.110*
Pancreas	0.365	0.304	0.225	0.152	0.095
Liver	0.149	0.148	0.163	0.135	0.064
Spleen	0.153	0.112	0.157	0.127	0.081
Kidney	0.153	0.122	0.098	0.098	0.047
Lung	0.126	0.057†	0.058	0.065†	0.045†
Heart	0.076	0.049	0.042	0.042	0.021
Thymus	0.304	0.319	0.190		0.046*

* Average of two specimens available for analysis in this group.

† Average of six specimens available for analysis.

The average values given in Table I are grouped according to age, in consideration of differences common in dietary practice at different ages and also in view of differences in metabolism. Presumably the vitamin content of the tissues is primarily dependent upon dietary intake, but may be further varied to a lesser extent in response to physiological disturbances. Phillips and Chang (6) have found a moderate increase in the vitamin C content of rat tissues as a result of chronic fluoride poisoning, apparently as a compensating factor in tissue respiration (the rat being capable of vitamin C synthesis).

In Table II an indication is given of the degree of variation found for different individuals in the same age groups. It is reasonable to conclude that, in cases in which the vitamin content was extremely low, there had existed a condition of latent scurvy.

TABLE II

Typical Individual Variations in Vitamin C Content of Tissues (Mg. per Gm.)

Age	Adrenal	Pancreas	Liver	Spleen	Kidney	Heart
14 days	0.913	0.237	0.244	0.126	0.200	0.076
Still-born	0.745	0.343	0.110	0.184	0.099	0.042
24 days	0.059	0.056	0.078	0.018	0.047	0.020
3 mos.	1.300	1.000	0.238	0.188	0.305	0.117
11 "	0.084	0.084	0.068	0.048	0.034	0.017
5 yrs.	1.030	0.274	0.234	0.161	0.145	0.078
5 "	0.191	0.087	0.114	0.124	0.067	0.049
64 "	0.706	0.177	0.276	0.272	0.040	0.053
55 "	0.033	0.022	0.029	0.023	0.022	0.027
70 "	0.027	0.022	0.018	0.012	0.011	0.017

TABLE III

Depletion of Vitamin C from Guinea Pig Tissues

Diet	Adrenal	Liver	Kidney	No. of animals
	mg. per gm.	*mg. per gm.*	*mg. per gm.*	
Basal + spinach*	0.70	0.10	0.08	8
" vitamin C-free, 15 days (beginning to lose weight)	0.08	0.03	0.03	6
Basal vitamin C-free, 28 days (severe scurvy)	0.03	0.01	0.01	6

* These animals are not necessarily normal in the sense of having an optimum vitamin intake, for animals having the vitamin under physiological control maintain a concentration approximately 100 per cent higher (1).

Of the thirty-one cases less than 10 years of age, four individuals were in a range so low that latent scurvy was clearly evident. Others were in an intermediate range indicated by the extreme values given in Table II. Among the thirty-six cases over 10 years of age, in six individuals the vitamin contents of the tissues were critically low, indicating a depletion nearly as severe as found in terminal human or guinea pig scurvy. From a study of the case histories and autopsy findings, there had been no specific consideration of scurvy in any of the cases. There was evidence in several cases however that the patients had been upon dietaries very low in antiscorbutic foods for several weeks or more prior to admission to the hospitals, and there were several cases in which clinical and autopsy records indicated conditions which might well have been in part due to latent vitamin deficiency. Generalized infections were more common among those having a low vitamin C content in their tissues. It is suggested that a titration of the vitamin C content of blood samples or other body fluids might be of diagnostic value clinically, and that tissue titrations might be of value frequently as a supplement to autopsy records.

Table III gives comparative data for guinea pigs, which may serve to indicate the general distribution and rate of depletion in relation to the onset of scurvy.

The authors are greatly indebted to the several pathologists who cooperated in supplying the tissues for analysis and in discussing the physiological implications of the chemical data. The courtesies extended by the following hospitals are also gratefully acknowledged: Mercy, Children's, Western Pennsylvania, St. John's, Elizabeth Steele Magee.

SUMMARY

The quantities and relative distributions of vitamin C, found by direct chemical titration of human tissues from 67 hospital autopsies, were found to correspond fairly closely with data obtained from titration of guinea pig tissues. The general order according to concentration was: adrenal, brain, pancreas, liver, spleen, kidney, lung, heart, muscle. In the younger age groups the thymus content was about as high as that of the pancreas. The average values ranged from about 0.55 mg. per gm. for adrenal tissue down to about 0.04 mg. for heart tissue. Individual cases varied from approximately 3 times higher than the average down to less than 0.1 the average values. The average for each of the tissues from those under 10 years of age was distinctly higher than from those over 10 years of age. Approximately 20 per cent of the cases gave evidence of a condition of latent scurvy.

BIBLIOGRAPHY

1. Bessey, O. A., and King, C. G., *J. Biol. Chem.*, **103**, 687 (1933).
2. Harris, L. J., Ray, S. N., and Ward, A., *Biochem. J.*, **27**, 2010 (1933).
3. Dalldorf, G., *Am. J. Dis. Child.*, **46**, 794 (1933).
4. Hanke, M. T., Diet and dental health, Chicago (1933).
5. Bessey, O. A., Menten, M. L., and King, C. G., *Proc. Soc. Exp. Biol. and Med.*, **31**, 455 (1934).
6. Phillips, P. H., and Chang, C. Y., *J. Biol. Chem.*, **105**, 405 (1934).

THE EFFECTS OF MODERATE DOSES OF VIOSTEROL AND OF PARATHYROID EXTRACT UPON RATS*

BY AGNES FAY MORGAN, LOUISE KIMMEL, RACHEL THOMAS, AND ZDENKA SAMISCH

(*From the Laboratory of Household Science, University of California, Berkeley*)

(Received for publication, April 4, 1934)

Much work has been done in recent years upon the toxic effects of overdoses of viosterol and of parathyroid extract in the effort to discern the mode of action of these potent substances. Shelling and Asher (18), Hess, Benjamin, and Gross (6), Jones and Robson (9), Harris and Innes (5), and others have tried the effects of varying the calcium and phosphorus contents of the experimental diets and have found that the pathological manifestations of hypervitaminosis are more obvious and severe usually on diets which provide generous amounts of these elements, although even calcium- and phosphorus-free diets may allow the symptoms to appear if a large enough excess of viosterol is used (6). The clinical problem, however, is most concerned with the limits of toxicity of this substance as well as that of parathyroid extract as they occur in diets of normal calcium and phosphorus content and ratio. It seemed worth while therefore to make a detailed study of the bones, soft tissues, and serum of rats fed such a diet and given varying and moderately excessive doses of viosterol and parathyroid extract both together and separately.

The diet used had the composition shown in Table I. This diet allows normal growth either with or without viosterol in therapeutic amounts. The rats which were used in the present study were placed on the diet at 28 days of age and during the following 6 to 8 weeks gained 18 gm. a week when given viosterol, 3 D or 10 D, and

---

* A brief report of this study was presented before the meeting of the American Society of Biological Chemists at Cincinnati, April 12, 1933 (*J. Biol. Chem.*, **100**, lxxi (1933); *Proc. Am. Soc. Biol. Chem.*, **8**, lxxi (1933)).

15 gm. a week without such additions. The calcium and phosphorus content and ratio, 0.62 and 0.40 per cent and 1.55 respectively, are close to the range usually suggested as optimal for rats. The Ca:P ratio is also similar to that of cow's milk, a food making up much of the diet of infants treated with viosterol. Six groups of animals were used altogether, four for comparison of effects of varying doses of viosterol[1] and two for observation of similar results of the injection of parathyroid extract.[2] The procedure consisted of feeding the diet to the rats beginning at the age of 28 days for 5 to 8 weeks, usually 6 weeks. Calcium and phosphorus balance studies were made on three of the groups during most of

TABLE I

Composition of Diet

	per cent
Wheat gluten	10
Alcohol-extracted egg albumin	10
Agar	2
Crisco	15
Corn-starch	59
Salt mixture*	4
Calcium	0.62
Phosphorus	0.40
Ca:P ratio, 1.55	
	mg. per rat per day
Supplement (these were fed as sources of vitamins B, G, and A)	
Yeast Vitamine-Harris	50
Carotene in ethyl laurate	0.006

* The composition of the salt mixture is as follows:

K_2HPO_4	18.5	KI	0.005
$Ca(H_2PO_4)_2$	51.2	NaF	0.062
$MgSO_4$	4.5	$MnSO_4$	0.019
NaCl	20.4	$KAl(SO_4)_2$	0.006
Fe citrate	2.0	Ca lactate	89.7

---

[1] We are indebted to Mead Johnson and Company through Dr. C. E. Bills for the viosterol 10,000 × which was used in these experiments.

[2] We are indebted to E. R. Squibb and Sons for the parathyroid extract used in this part of the study.

this time. At the end of the feeding period the animals were killed and blood was removed without anticoagulant from the beating heart. Calcium and inorganic phosphorus were determined in the serum. Kidneys and usually hearts as well as femurs and tibias were dissected out, dried, and analyzed for ash, calcium, and phosphorus. The bones were extracted thoroughly with alcohol and ether before being incinerated and were divided according to the suggestion of Hess, Berliner, and Weinstock (7) into portions consisting of metaphyses, or the spongy epiphyseal portion, and shafts, or diaphyses. These parts were ashed separately. However, only the epiphyseal portion of the tibias was examined. The line test technique was applied in most cases to one of the tibias and a record made of the amount of spongiosa seen under the binocular microscope.

Calcium and Phosphorus Retentions—As may be seen in Table II, the retention of calcium on this diet is favorably affected by viosterol fed at the level of 10 D. Throughout our study we have used the Steenbock vitamin D unit to designate the amount of irradiated ergosterol given. The commercial 250 D preparation used at first was tested and found to contain the full potency indicated. The first three groups of rats used were divided into two parts, one-half receiving no addition of viosterol and one-half a corn oil dilution of the viosterol equivalent to 10 times the therapeutic dose of cod liver oil.

Eight balance studies of 1 or 2 weeks duration each were made upon these six groups, during two of which 80 units of parathyroid extract were injected in four doses into each of the rats. In all but one of the balance studies, which were uncomplicated by parathyroid treatment, the calcium retentions of the viosterol-fed rats were considerably greater, ranging from 26 to 39 per cent more than were those of the group given no viosterol. The phosphorus retentions during these periods were usually but not invariably better in the viosterol-fed group. This more specific effect upon calcium retention is reflected in the larger Ca:P ratio of retentions of the viosterol-fed animals.

During Periods 2 and 4 for Group II-b, in which 80 units of parathyroid extract were administered, the excretion of both calcium and phosphorus was increased in both viosterol-fed animals and those not fed viosterol. In Period 2, moreover, the intakes of these elements were only slightly depressed below those seen in Periods 1 and 3 for the same animals without parathyroid treatment. During Period 2 the retention of calcium by the viosterol-fed rats was reduced by about 53 per cent from that of the comparable Periods 1 and 3 while the calcium retention of the rats without viosterol was reduced by 35 per cent. The corresponding reductions in phosphorus retention during Period 2 from the averages for Periods 1 and 3 were 49 and 40 per cent. The influence of the viosterol, if any exists, on the losses occasioned by parathyroid treatment is to increase such losses.

TABLE II

Calcium and Phosphorus Retentions of Rats with and without Viosterol and Parathyroid Extract (Mg. per Rat per Week)

Group No	No. of rats	Age at beginning	Balance studies		Viosterol	Total parathyroid extract	Calcium			Phosphorus		
			Period No.	Duration			Intake	Output	Retention	Intake	Output	Retention
		days		*days*		*units*						
I	6	49	1	14	10 D		293	116	177	190	78	112
	8				None		244	156	88	163	94	69
II-a	6	28	1	14	10 D		229	102	127	145	65	80
	6				None		229	118	111	145	58	87
	6	42	2	7	10 D		227	130	97	142	68	74
	8				None		228	158	70	144	82	62
	6	49	3	14	10 D		250	149	101	157	87	70
	7				None		248	127	121	157	76	81
II-b	5	28	1	14	10 D		244	105	139	158	60	98
	7				None		243	142	101	158	76	82
	3	42	2	7	10 D	80	213	148	65	140	95	45
	8				None	80	220	149	71	144	96	48
	3	49	3	14	10 D		239	102	137	155	76	79
	8				None		240	123	117	156	77	79
	7	63	4	7	10 D	80	120	89	31	83	71	12
	8				None	80	167	121	46	111	78	33

The changes in the retentions due to parathyroid treatment in Period 4 of Group II-b are less easily assessed because of the greater lowering of food intake due to the effect of the parathyroid extract.

It is obvious, however, that the retentions of both calcium and phosphorus are smaller in proportion to intakes in this period than in the non-parathyroid Periods 1 and 3. Thus in Periods 1 and 3 of Group II-b the percentage of calcium intake retained is 41 to 57 and of phosphorus is 51 to 62. In Period 2 it is 30 to 33 per cent of both calcium and phosphorus intakes and in Period 4, 14 to 29 per cent.

Thus the primary effect of the viosterol appears to be upon calcium retention, perhaps through a decrease in the ease of calcium excretion into the intestine but without so consistent an effect upon phosphorus output. The parathyroid extract on the other hand appears to act both upon phosphorus and calcium outputs, increasing them in both cases. The calcium output is increased more significantly by the parathyroid treatment in the case of the rats receiving viosterol than in those without it. This raises some question as to the validity of the hypothesis that the hormone acts only by stripping calcium phosphate from the bones. If circulating calcium is less easily excreted in the presence of vitamin D as is often predicated, a less noticeable increase in output should be seen following the administration of the extract to the rats receiving viosterol than to those which did not receive it. But this is apparently not the case.

The study of Pugsley (16) upon calcium and phosphorus output as affected by parathyroid extract was not parallel with ours, since he used adult rats fed a low calcium diet, 0.266 per cent phosphorus, 0.025 per cent calcium, of very low Ca:P ratio, and obtained in consequence the typical immunity or gradual failure of response to the hormone which we have found (15) associated only with the low calcium diet. He noted an increase at first in phosphorus and calcium excretion in the parathyroid-treated animals, followed by a decrease in most cases, but made no attempt at calculating gross retentions.

Serum Calcium and Phosphorus—The anticipated larger increase in serum calcium due to parathyroid extract injection in the presence of vitamin D as compared with the condition in its absence, is actually seen in the serum of our rats which also received viosterol. Table III shows that the serum phosphate is little changed by this treatment in any of the groups on this diet, but serum calcium is more easily raised by parathyroid extract in the groups receiving 10 D than in those without it. Thus 60 units of the extract raised the serum calcium of the rats fed viosterol 10 D from 12.0 to 15.5 mg. per 100 cc., but had no effect on the serum calcium of the rats without viosterol. However, 160 units raised both serum calcium and phosphorus of the latter group to the high levels of 28.7 and 14.3 mg. per 100 cc. respectively. The same amount of the hormone administered to rats given viosterol 10 D was fatal in nearly all cases and the blood was so dehydrated and viscous as to prove impossible to analyze. Thus the effects of the vitamin and the hormone appear to be similar and cumulative. The less notable changes in serum inorganic P may be due to the greater ease with which this substance is excreted by way of the kidneys. This was not verified in our study, however, because no separation of output in urine and feces was attempted in the balance experiments.

TABLE III

Serum Calcium and Phosphorus of Rats 63 to 67 Days Old As Affected by Viosterol and Parathyroid Extract

No. of groups	Total No. of rats	Viosterol	Total parathyroid extract	Total Ca	Inorganic P
			units	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*
8	24	None		12.0	11.0
4	17	"		12.2	8.9
1	6	"	160	28.7	14.3
4	11	"	60	11.6	11.0
5	19	3 D		13.3	9.4
7	22	10 D		12.0	10.1
3	6	10 D	160		
2	8	10 D	60	15.5	11.1
2	8	500 D		13.6	9.0
5	18	1000 D		15.7	12.8
3	9	1500 D		14.5	12.0
3	8	2000 D		17.2	12.2

The larger doses of viosterol were given as dilutions of the viosterol 10,000 × supplied by Dr. C. E. Bills. This was taken to be equivalent to 30,000 D in Steenbock units. Serum analyses were made on rats given viosterol 500 D, 1000 D, 1500 D, and 2000 D. As shown in Table III a significant rise in calcium is produced at

1000 D, 1500 D, and 2000 D levels. This rise is of the order of that produced by 60 units of parathyroid extract plus viosterol 10 D.

Calcification of Kidneys and Hearts—The ash, calcium, and phosphorus contents of dried kidneys in all cases and dry blood-free hearts in some cases were determined. Table IV shows the striking increase in calcium and phosphorus content of these organs produced by even apparently innocuous doses of parathyroid extract. In only one group, that of the non-viosterol-fed rats given 60 units, was a normal content found in the hearts and in no cases in the kidneys. On the other hand moderate excess of viosterol alone produces considerably less mineralization of the kidneys, although from 1000 D up abnormal amounts of both calcium and phosphorus were present. In the case of two rats which by mistake were given viosterol 4000 D for 1 week and which died of this overdosage, very high ash content of the kidneys was found. The six rats given viosterol 10 D and 80 or 100 units of parathyroid extract also died of overdosage and these are seen to have also

TABLE IV

Ash, Calcium, and Phosphorus Content (Measured in Per Cent of Dry Weight) of Kidney and Heart of Rats As Affected by Moderate Amounts of Viosterol and Parathyroid Extract

No. of groups	No. of rats	Age	Viosterol	Total parathyroid extract	Kidney				Heart			
					Ash	Ca	P	Ca:P	Ash	Ca	P	Ca:P
		days		*units*								
6	35	68	None		4.3	0.08	0.87	0.09	4.4	0.05	0.59	0.08
2	19	64	3 D		4.5	0.08	0.94	0.08				
3	12	70	10 D		4.2	0.05	0.82	0.06	4.5	0.06	0.49	0.12
1	8	64	500 D		4.4	0.10	1.02	0.09				
2	18	64	1000 D		5.3	0.13	1.13	0.11				
1	9	64	1500 D		4.9	0.19	1.10	0.18				
1	8	64	2000 D		6.0	0.32	1.25	0.25				
1	2		4000 D			7.64	5.29	1.44				
1	3	70	10 D	160		4.53	2.60	1.36		1.10	0.60	1.83
2	6	60	10 D	80–100		5.16	2.81	1.83		0.68	0.80	0.85
5	13	70	10 D	60	10.1	3.09	2.14	1.44	5.4	0.45	0.92	0.49
1	7	70	None	160		5.58	2.74	2.00		1.15	0.67	1.71
3	9	70	"	60	9.1	1.91	1.87	1.02	5.0	0.08	0.59	0.13

exceedingly high calcium and phosphorus content of kidneys. Apparently surviving animals are able to demineralize these organs to some degree, and death from overdosage either of the extract or the viosterol may be due to renal failure from this calcification. Attention might well be paid to this possibility in the clinical use of both parathyroid extract and viosterol.

The far greater deposition of these elements in the kidneys of the parathyroid-treated animals as compared with those receiving moderate excesses of viosterol is puzzling in view of the fact that the serum values for calcium and phosphorus were even greater in the latter in some cases than in the former. The partition of the serum calcium between diffusible and non-diffusible fractions may be different in the two groups, but no clear demonstration of this is as yet available.

This effect of the parathyroid extract on the kidneys was observed by Hueper (8) and has been suggested as the probable cause of death from overdosage. Light, Miller, and Frey (12) offer data on the kidney and heart ash of their animals poisoned by excessive doses of viosterol which are difficult to interpret and apparently erroneous since the calcium and phosphorus content and ratio of the ashes are quite unlike any others reported in the literature. Smith and Elvove (19) found calcium contents in the kidneys of rabbits given 2.5 and 10 mg. of irradiated ergosterol daily, which are of much the same order as those seen in our parathyroid-treated rats. Brown and Shohl (3) noted metastatic calcification in rats beginning at the 5000 D level and Shelling and Asher (18) at 1250 D in animals on a stock diet quite like ours in calcium and phosphorus content. Actual analyses are offered only by Light, Miller, and Frey and by Smith and Elvove.

Composition of Bones—The separate analyses of the metaphyses and diaphyses of the femurs revealed certain differences not discernible by ashing of the whole bone. The spongiosa-containing portion of the tibias was also examined and this was found to be practically the same in ash, calcium, and phosphorus content as the corresponding part of the femurs. As seen in Table V little difference exists in the ash per cent of extracted tibias, metaphyses, and shafts of rats given this normal diet and no viosterol or doses at the levels of 3 D and 10 D. The average ash values are 49.1 for the tibias, 49.9 for the metaphyses, and 63.5 for the shafts.

There is no significant difference from these averages exhibited by any of the groups on these three régimes. The group given viosterol 500 D appears to have a definite hypercalcification, however, the metaphyses being 52.7 per cent ash, although the shafts are about like the others, that is 64.1 per cent ash. The groups given viosterol 1000 D, 1500 D, and 2000 D show significant and progressive lowering of the ash of both metaphyses and shafts in about the same amounts.

The animals given 60 to 160 units of parathyroid extract with no viosterol exhibited no effect of the treatment in the bones, but those which received the same amounts of hormone along with viosterol 10 D had a distinct lowering of the metaphysis ash of the same order as that seen in the rats given viosterol 1000 D. However, no lowering of ash content of the shafts was seen. It is possible that larger doses or a longer treatment with the parathyroid extract might produce a demineralization of the shafts. It is interesting, however, to note the rapid depletion of the trabecular ash produced without thinning of the cortex by the extract as compared with similar losses due to 1000 D viosterol dosage, for example, which was accompanied by definite demineralization of the shafts.

Table V

Ash Content of Fractions of Bones of Rats, 64 to 84 Days Old, As Affected by Viosterol and Parathyroid Extract

No. of groups	No. of rats	Viosterol	Total parathyroid extract	Ash in extracted bones			
				Tibia	Metaphysis of femur	Shaft of femur	Whole femur
			units	*per cent*	*per cent*	*per cent*	*per cent*
5	42			47.9	49.8 ± 0.3	63.4 ± 0.2	57.1
2	18		60–160	47.1	48.2 ± 0.5	64.0 ± 0.3	56.8
2	19	3 D		51.2	49.5 ± 0.3	63.1 ± 0.4	
3	20	10 D		48.3	50.5 ± 1.2	64.1 ± 0.4	57.4
4	23	10 D	60–160	45.1	46.9 ± 0.5	63.5 ± 0.3	56.7
1	8	500 D		50.5	52.7 ± 0.4	64.1 ± 0.2	
2	18	1000 D		47.2	46.6 ± 0.2	60.0 ± 0.2	
1	9	1500 D		48.1	45.9 ± 0.6	61.9 ± 0.3	
1	8	2000 D		44.1	44.5 ± 0.4	57.8 ± 0.3	

Calcium and phosphorus determinations were made upon the ash of most of the bone fractions examined. Except for certain irregularities in the proportions of these elements in the ash of the tibias, almost constant figures were found. Ash of metaphyses and shafts of the femurs in all cases contained 36.8 to 39.8 per cent calcium and 16.3 to 18.6 per cent phosphorus, the means being 38.0 and 17.6. These are close to the theoretical figures, 38.9 and 17.2, calculated from the dahllite formula for bone, $CaCO_3 \cdot 2Ca_3(PO_4)_2$. Greater variability is seen in the corresponding proportions in the tibias. Whatever the nature of the ash loss in the bones of animals given excess viosterol and parathyroid extract, it does not involve any notable change in the proportions of calcium and phosphorus. This confirms a number of previous observations, among which the x-ray studies of Taylor and Sheard (20) and Roseberry, Hastings, and Morse (17) may be mentioned as indicating a definite crystalline structure of bone. Dislodgement of both carbonate and phosphate simultaneously must occur in the dissolution of bone brought about by both the excess viosterol and parathyroid extract.

Appearance of Cut Bones—It has been noted previously (13) that this normal diet without vitamin D produces bones of normal strength but unusually rich in well calcified spongiosa. Ash analyses do not indicate that these bones are hypercalcified, although the metaphyses often appear richer in ash and in calcium than do those of similar animals fed optimal amounts of vitamin D. The orderly and rather scanty trabeculæ shown by rats given viosterol are depleted in the bone of the parathyroid-treated animals as are the more profuse deposits seen in rats without vitamin D when similarly treated.

In Fig. 1 are shown illustrative cut tibias treated with silver nitrate in order to darken the calcified areas. These are from three rats fed the normal diet, one without vitamin D, one with viosterol 3 D, and one with viosterol 1000 D. The heavy trabeculæ characteristic of no vitamin D are clearly evident, the fine regular calcium line of the bone of animals receiving normal doses of vitamin D, and an irregular and thinning deposit in the bone of those receiving viosterol 1000 D. Harris and Innes (5) have described as due to excess viosterol a depletion of the hypercalcified spongiosa beginning at the epiphyseal growth cartilage and moving toward the marrow cavity. We have seen but little intermediary spongiosa calcification even in the group receiving viosterol 500 D and cannot interpret the appearance of the bones of animals receiving viosterol 1000 D and viosterol 2000 D as representing the progressive depletion of such deposits. The break in the center of the growth cartilage in these bones is characteristic and has been described also by Göttsche and Kellner (4). The second line of trabeculæ apparently receding from the epiphyseal growth area may represent merely the partly completed process of resorption of bony material both from the epiphyseal border and from the cortex of the shaft. Our ash figures seem to indicate that both parts of the bone are simultaneously and equally decalcified by excess vitamin D. The apparently clean removal of trabeculæ at the growth cartilage which we have seen in these and previous studies (13) of parathyroid-treated bones may mean that moderate doses of the hormone first cause resorption of the trabecular deposit and perhaps only in excessive amounts attack the cortex. This is indicated by our figures for ash (Table V).

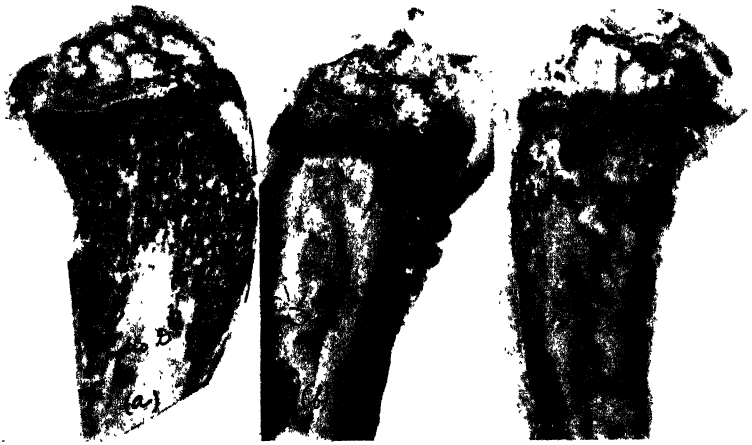


Fig. 1. The appearance of cut tibias of rats 65 days old, fed a normal diet, (*a*) from a rat which received no vitamin D, (*b*) from a rat which received viosterol 3 D, and (*c*) from a rat which received viosterol 1000 D.

Hess, Berliner, and Weinstock (7) found for eleven rats on normal diet values of 63.8 per cent ash in the shafts and 51.2 in the metaphyses. These figures are very close to those shown by our normals. Brown and Shohl (3) saw a rise in total femur ash with viosterol 100 D and 1000 D but a fall from 5000 D on. Light, Miller, and Frey (12) noted a fall in femur ash beginning at 40 D. Jones and Robson (10) have recently described a similar fall in femur ash on 30,000 D. Bischoff (2) using dogs, Bauer, Aub, and Albright (1) using rats, and Taylor, Weld, Branion, and Kay (21) using fowls, found denser bones resulting from parathyroid treatment. But Lambie, Kermack, and Harvey (11) in rats, and Bauer, Aub, and Albright (1) in rabbits have observed thinning of trabeculæ from the same treatment. It is possible that both these conditions may result even in the same animal if depletion of trabeculæ be followed at first by deposition in the shaft of the calcium phosphate thus removed.

In any case, all of the effects noted by us as resulting from the parathyroid treatment are more obvious in those animals which also received the moderate dose of viosterol, 10 D, than in those which received no viosterol. Thus the calcium and phosphorus retentions were more reduced on the same intake when parathyroid was given the viosterol-fed group, the serum calcium was more elevated in the cases which could be compared, the kidneys and hearts contained more calcium phosphate, the bone ash was more reduced in the viosterol-fed than in the non-viosterol-fed rats. Quite comparable effects have been observed in our laboratory previously on rats and on dogs (14).

If a distinction between the mode of action of these two agencies exists, the parathyroid hormone and vitamin D, it can only be discovered by seeking for clear differences in their effects. So far such differences have not been satisfactorily demonstrated. Such differences as we have here noted may be only matters of degree rather than of kind. The greater calcification of kidneys and hearts produced by the parathyroid extract with a given rise in serum calcium and phosphate as compared with that produced by excess viosterol, may possibly be achieved by the latter in larger concentrations. Indeed, the calcification of kidneys of our two rats which died of 4000 D viosterol appears to illustrate this fact.

The more specific depletion of trabeculæ rather than of shafts which our parathyroid-treated rats show may possibly be duplicated by excess viosterol under other circumstances than those of our experiments.

SUMMARY

1. The retention of calcium and phosphorus was usually better in rats fed a diet of normal calcium and phosphorus content and ratio with viosterol 10 D than without it. When 80 units of parathyroid extract were given each animal during 1 week the calcium and phosphorus retentions were lowered in both cases but more noticeably in the viosterol-fed group.

2. The serum calcium and inorganic phosphate were more increased by parathyroid extract in the rats which received viosterol 10 D than in those which did not and were raised in proportion to the amount of the extract given. Viosterol 1000 D and 1500 D had an effect on serum similar to that of viosterol 10 D with 60 units of parathyroid extract.

3. There was a progressive increase in ash, calcium, and phosphorus content of kidneys with increasing doses of viosterol up to 2000 D and a similar but far greater increase in the parathyroid-treated animals. Kidney ash in the latter was raised 2 to 3 times the normal values.

4. The metaphysis or head of the femurs and tibias and also the shafts showed progressive loss of ash with increasing amounts of viosterol, 500 D to 2000 D, but the parathyroid treatment produced little ash depletion, particularly in the shafts.

5. The cut tibias showed heavy trabecular deposit in the rats given no vitamin D, a fine clear line of calcium deposition at the epiphyseal margin in those given normal doses of vitamin D, an irregular thin deposit with break in cartilage and secondary deposit in the marrow in those given moderately excessive doses of viosterol. The calcium and phosphorus content of all bone ashes remained within the normal range.

Thus viosterol and parathyroid extract have similar and additive effects upon serum, kidneys, and bones of animals fed the normal diet but the calcification of kidneys is more marked in the parathyroid-treated and decalcification of bones more advanced in the viosterol-treated animals of similar serum composition.

BIBLIOGRAPHY

1. Bauer, W., Aub, J. C., and Albright, F., *J. Exp. Med.*, **49**, 145 (1929).
2. Bischoff, G., *Z. ges. exp. Med.*, **68**, 772 (1929).
3. Brown, H. B., and Shohl, A. T., *J. Biol. Chem.*, **86**, 245 (1930).
4. Göttsche, O., and Kellner, B., *Arch. Kinderh.*, **97**, 76 (1932).
5. Harris, L. J., and Innes, J. R. M., *Biochem. J.*, **25**, 367 (1931).
6. Hess, A. F., Benjamin, H. R., and Gross, J., *J. Biol. Chem.*, **94**, 1 (1931–32).
7. Hess, A. F., Berliner, F. S., and Weinstock, M., *J. Biol. Chem.*, **94**, 9 (1931–32).
8. Hueper, W. C., *Arch. Path. and Lab. Med.*, **3**, 14, 1002 (1927).
9. Jones, J. H., and Robson, G. M., *J. Biol. Chem.*, **91**, 43 (1931).
10. Jones, J. H., and Robson, G. M., *Am. J. Physiol.*, **103**, 338 (1933).
11. Lambie, C. G., Kermack, W. O., and Harvey, W. F., *Nature*, **123**, 348 (1929).
12. Light, R. F., Miller, G., and Frey, C. N., *J. Biol. Chem.*, **84**, 487 (1929).
13. Morgan, A. F., and Field, J. G., *Am. J. Physiol.*, **105**, 585 (1933).
14. Morgan, A. F., and Garrison, E. A., *J. Biol. Chem.*, **85**, 687 (1929–30); *Am. J. Physiol.*, **105**, 596 (1933).
15. Morgan, A. F., Garrison, E. A., and Hills, M. J., *Am. J. Physiol.*, **105**, 608 (1933).
16. Pugsley, L. I., *J. Physiol.*, **76**, 21 (1932).
17. Roseberry, H. H., Hastings, A. B., and Morse, J. K., *J. Biol. Chem.*, **90**, 395 (1931).
18. Shelling, D. H., and Asher, D. E., *Bull. Johns Hopkins Hosp.*, **50**, 318 (1932).
19. Smith, M. I., and Elvove, E., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1245 (1929).
20. Taylor, N. W., and Sheard, C., *J. Biol. Chem.*, **81**, 479 (1929).
21. Taylor, N. B., Weld, C. B., Branion, H. D., and Kay, H. D., *Canad. Med. Assn. J.*, **24**, 763 (1931); **25**, 20 (1931).

THE EFFECT OF ACIDITY ON THE CARBON MONOXIDE-COMBINING POWER OF HEMOGLOBIN IN THE BLOOD OF MARINE FISHES*

BY RAYMOND W. ROOT AND ARDA ALDEN GREEN

(*From the Woods Hole Oceanographic Institution, Woods Hole*)

(Received for publication, June 21, 1934)

Previous studies on the effect of hydrogen ions on the equilibrium between hemoglobin and oxygen in the blood of a number of marine teleosts (Root, 1931; Green and Root, 1933) indicated rather strongly that acidity not only exerts the usual Bohr effect on these hemoglobins, but also causes them to lose a large fraction of their oxygen-combining power, even when they are exposed to an atmosphere of oxygen. It seemed probable, from the shape of the dissociation curves at acid reactions, that increasing the oxygen tension still further would not result in increasing the amount of oxygen combined with the hemoglobin—that is, that there is a true reversible inactivation of the respiratory pigment. Such inactivation might be due to the inability of the prosthetic group on the hemoglobin to combine with any gas in the presence of acid, or depend upon some particular behavior of the oxygen.

In human blood the dissociation curves for carboxyhemoglobin are essentially like those for oxyhemoglobin, except for the fact that carbon monoxide has a combining power some 245 times greater than that of oxygen (Douglas, Haldane, and Haldane, 1912). If such a situation exists with respect to fish hemoglobin, then data procured with the use of carbon monoxide ought to be comparable with data obtained by raising the oxygen tension by an amount proportional to the difference in combining power of the two gases, and, thus, show whether an increase in oxygen tension far above that used in previous work would increase the amount of oxygen bound in acid solutions. With this idea in mind a comparison has been made of the behavior of fish hemoglobin in the presence of oxygen and carbon monoxide, especially at acid reactions.

---

* Contribution No. 50.

Experiments were conducted with the bloods of the sea-robin, *Prionotus carolinus*, L., the tautog, *Tautoga onitis*, L., and the toadfish, *Opsanus tau*, L. The results show that there is no loss of carbon monoxide capacity in acid bloods. Moreover, the use of carbon monoxide is not exactly comparable with increasing the oxygen tension in proportion to the difference in their combining power, for the dissociation curves obtained with the two gases are not always similar in shape.

Methods

The technique of handling the blood was the same as described in the previous paper (Green and Root, 1933), except for the added procedure for the determination of carbon monoxide. All blood samples were equilibrated in a water bath at 25° and analyzed immediately in duplicate with the Van Slyke constant volume apparatus. The procedure described by Sendroy and Liu (1930) was used for the determination of carbon monoxide in the blood, the gas finally being absorbed by ammoniacal cuprous chloride solution. The gas in the tonometers was analyzed with the Haldane apparatus to which was attached a combustion chamber for the determination of carbon monoxide. The carbon monoxide was prepared from formic and sulfuric acids and stored for use over water.

All data expressing the amount of gas in the blood have been corrected for dissolved gas. An oxygen solubility coefficient of 2.70 volumes per cent per atmosphere (Green and Root, 1933) was used in calculating dissolved oxygen. Carbon monoxide solubility coefficients were experimentally determined for sea-robin and tautog bloods, the values obtained being 1.75 and 2.09 volumes per cent per atmosphere respectively. The value for tautog blood was assumed to hold for toadfish blood.

The pH of the blood has been calculated from gasometric data, the Henderson-Hasselbalch equation being used, assuming pK'_1 equal to 6.22 for whole blood at 25°. Bohr's (1905) solubility coefficient was adopted for the determination of dissolved carbon dioxide. In a number of cases the pH was measured with the glass electrode. The values obtained by measurement averaged about 0.06 pH higher than the calculated values and indicate that some adjustment should be made in the constants of the equation. However, since constancy of pH is more important than the absolute values in this problem, no attempt has been made to evaluate new constants. The calculated pH value is given in each case, thus making it directly comparable with previous data on these bloods.

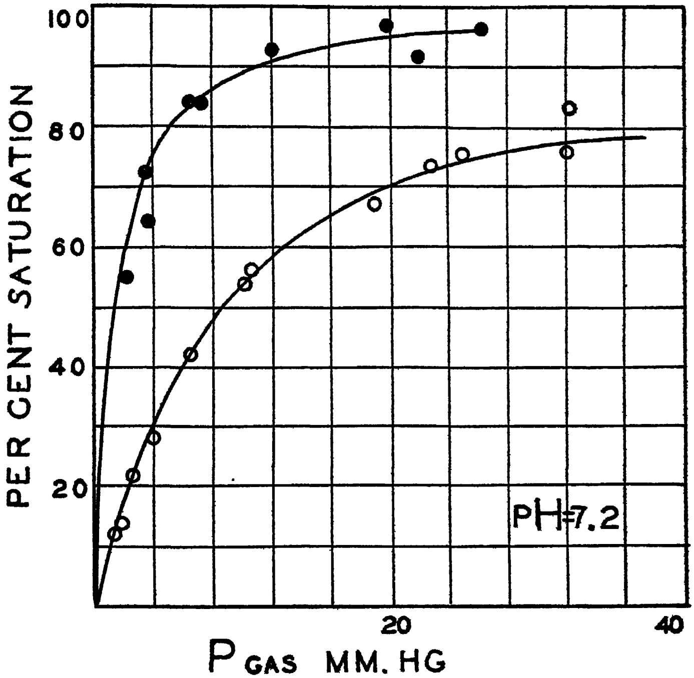


Fig. 1. The carbon monoxide (upper) and oxygen (lower) dissociation curves for sea-robin blood at pH 7.2. The oxygen pressure for each point on the oxygen curve has been divided by 25. The curves drawn through the points are rectangular hyperbolas. The dissociation constant for the carbon monoxide is actually 165 times greater than that for the oxygen curve.

Carbon Monoxide and Oxygen Dissociation Curves for Sea-Robin Blood

Because no data were available for the oxygen dissociation curves at 25°, both oxygen and carbon monoxide curves were established at comparable pH values for this blood. The data are summarized in Figs. 1 to 4.

The curves established at pH 7.2 are similar, both capable of being described by rectangular hyperbolas with no reversible inactivation of the hemoglobin. The constant for the carbon monoxide dissociation curve is about 165 times greater than that for the oxygen dissociation curve. From the data it would seem that sea-robin blood at this pH behaves in a manner similar to human blood.

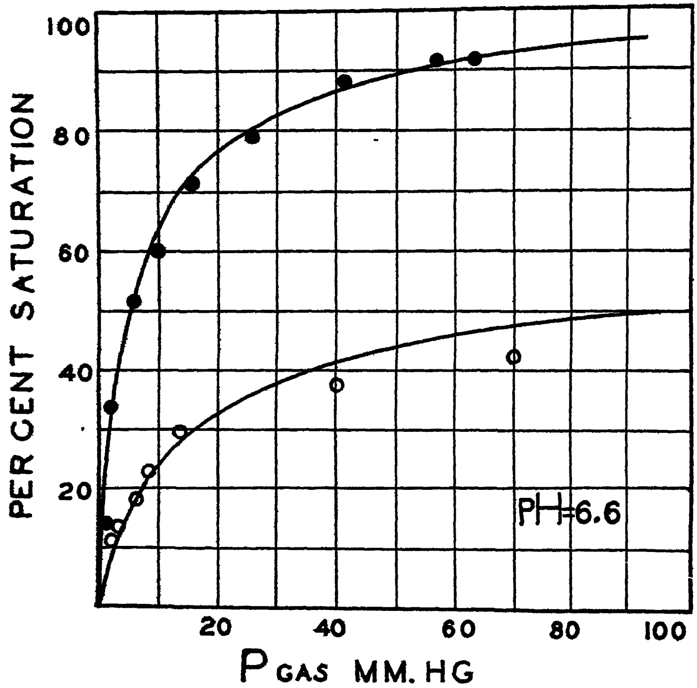


FIG. 2. The carbon monoxide (upper) and oxygen (lower) dissociation curves for sea-robin blood at pH 6.6. The oxygen pressure for each point on the oxygen curve has been divided by 10. The curves drawn through the points are rectangular hyperbolas with 50 per cent inactivation of hemoglobin assumed for the oxygen curve.

In more acid ranges the oxygen dissociation curves exhibit the apparent decrease in oxygen capacity described for several other fish bloods. The carbon monoxide dissociation curves, on the other hand, at comparable pH values do not show this phenomenon to any appreciable extent. Furthermore, the two sets of curves are definitely different. For example, at pH 6.6, even though both curves can be satisfactorily described by rectangular hyperbolas, the oxygen curve can only be so described by assuming that the hemoglobin is 50 per cent inactivated. When the blood is made still more acid (pH 6.3), the carbon monoxide capacity of the hemoglobin is unchanged, whereas apparently only 25 per cent of the normal oxygen capacity remains. Even at pH 5.5 the blood is 85 or 90 per cent saturated with carbon monoxide at 500 mm.

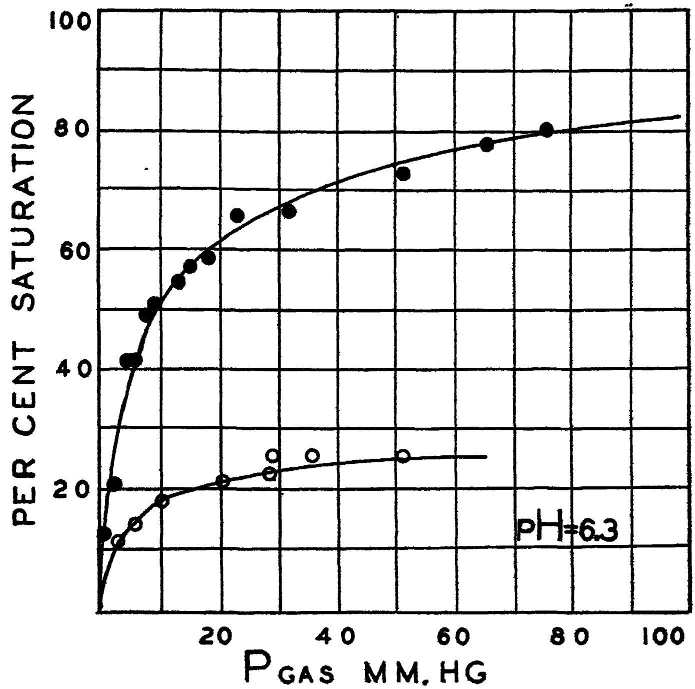


FIG. 3. The carbon monoxide (upper) and oxygen (lower) dissociation curves for sea-robin blood at pH 6.3. The oxygen pressure for each point on the oxygen curve has been divided by 10. A smooth curve not quite equal to a rectangular hyperbola passes through the data for the carbon monoxide dissociation. The lower curve is a rectangular hyperbola with 75 per cent inactivation of the hemoglobin assumed.

pressure, and the shape of the curve is such as to suggest that the blood would become completely saturated if the carbon monoxide tension were made greater.

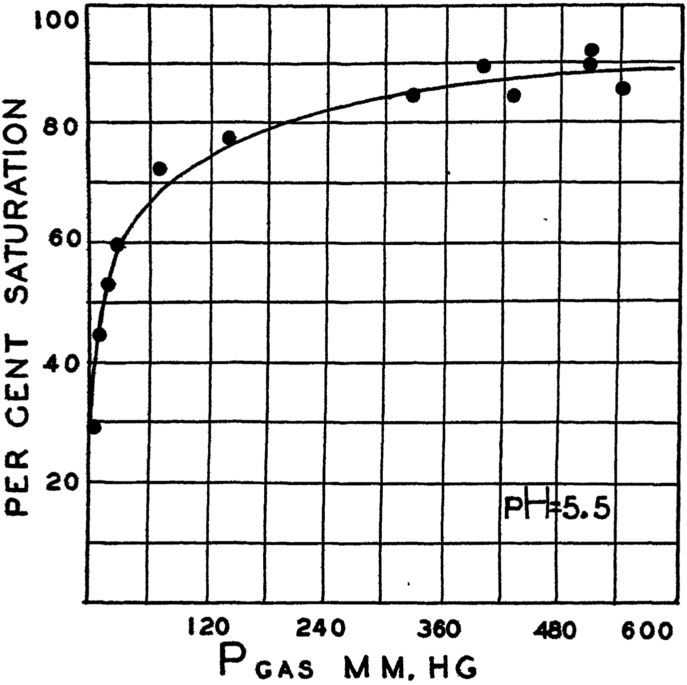


FIG. 4. The carbon monoxide curve for sea-robin blood at pH 5.5. A smooth curve not quite equal to a rectangular hyperbola passes through the points.

Carbon Monoxide Dissociation Curves for Toadfish and Tautog Bloods

Only one carbon monoxide curve was established for each of these bloods. The pH at which each curve was established is approximately 6.5, which is the acidity at which the oxygen dissociation curves indicate that approximately 50 per cent of the hemoglobin is inactivated (Green and Root, 1933). The data for the carbon monoxide dissociation curves are presented in Fig. 5. The position of the oxygen curves at the same pH is also shown in the figure for the sake of comparison.

By inspection of the carbon monoxide curves it becomes apparent that there is no reversible inactivation of the hemoglobins as far as carbon monoxide is concerned. A situation comparable to that described for sea-robin blood appears to be the case, for the oxygen and carbon monoxide curves are quite different. In order to fit the oxygen curves with the usual equations it is necessary to assume inactivation, whereas this is obviously unnecessary in the case of the carbon monoxide dissociation curves.

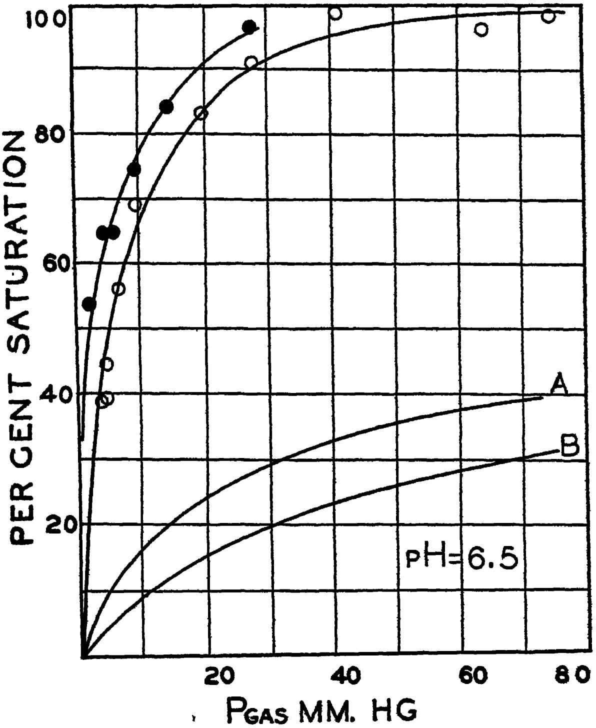


FIG. 5. The carbon monoxide dissociation curves for toadfish (upper, with dots) and tautog (lower, with circles) bloods at pH 6.5. The two lower curves represent the position of the corresponding oxygen dissociation curves, Curve A being that for toadfish, and Curve B that for tautog blood.

We wish to express to Professor A. C. Redfield our sincere thanks for his helpful suggestions and criticisms. We also wish to thank Mr. Walter Buck for technical assistance.

SUMMARY

The behavior of fish hemoglobin toward carbon monoxide and oxygen has been compared. It differs in two respects.

1. The hemoglobin can be saturated with carbon monoxide regardless of the pH (within the limits set by the experiments), whereas at acid reactions oxygen apparently combines with only a portion of the hemoglobin present.
2. At acid reactions the dissociation curves at comparable pH values differ in shape.

BIBLIOGRAPHY

Bohr, C., *Skand. Arch. Physiol.*, **17**, 104 (1905).

Douglas, C. G., Haldane, J. B. S., and Haldane, J. S., *J. Physiol.*, **44**, 275 (1912).

Green, A. A., and Root, R. W., *Biol. Bull.*, **64**, 383 (1933).

Root, R. W., *Biol. Bull.*, **61**, 427 (1931).

Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **89**, 133 (1930).

STUDIES ON MAGNESIUM DEFICIENCY IN ANIMALS

V. CHANGES IN THE MINERAL METABOLISM OF ANIMALS FOLLOWING MAGNESIUM DEPRIVATION

BY H. D. KRUSE, MARGUERITE M. SCHMIDT,* AND E. V. McCOLLUM

(*From the Biochemical Laboratory, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore*)

(Received for publication, June 26, 1934)

It has been reported previously that a ration containing only 1.8 parts per million of magnesium, but adequate amounts of other constituents, leads to a local disturbance in which vasodilatation, hyperirritability, and generalized convulsions represent a new form of tetany (1, 2), and to a constitutional disturbance in which loss of weight indicates nutritive failure (3). Furthermore, a complete study of the chemical changes in the blood in this syndrome has revealed relevant changes which may be correlated respectively with the tetany and the nutritive failure (4). In short, the essentiality of magnesium in animal economy is an established fact. The distinctive symptomatology and blood changes, arising in the course of magnesium deficiency, stamp the condition as a specific and separate entity. But our knowledge of the mechanisms at work in the various bodily systems is far from complete.

Because calcium is very closely related, both chemically and physiologically, to magnesium—both of the elements belong to the alkaline earth group; a disturbance of either in the animal body leads to tetany; both are the principal cations present in bone; and an antagonism is said to exist between them in their effects on the nervous, osseous, and excretory systems—and because phosphorus is the usual anion associated in the body with calcium and magnesium, it was deemed appropriate to study the changes which the three elements undergo in magnesium deprivation with a view to the following considerations: (a) the excretion of calcium, magnesium, and phosphorus in absolute amounts, (b) the pathway of excretion of these elements, (c) the predication of a physiological antagonism between calcium and magnesium. Moreover, the excretion of ammonia, acids, and nitrogen was investigated with the 2-fold purpose of lending additional evidence to the differentiation of magnesium tetany from other forms and of ascertaining a possible alteration of the level of protein metabolism during nutritive failure.

---

* Presented by Marguerite M. Schmidt to the faculty of the School of Hygiene and Public Health at the Johns Hopkins University in partial fulfilment of the requirements for the degree of Doctor of Science in Hygiene.

Technique and Methods

Three young female dogs weighing 2.7 to 3 kilos, after a preliminary period of a week on a dry stock ration, such as is used in our rat colony, were confined to metabolism cages, two animals then receiving the diet containing only 1.8 parts per million of magnesium, while the third animal received this diet fortified with adequate magnesium. The composition of the diets has already been described (1). The animals had constant access to the diet and distilled water. To prevent scattering of the ration, it was found advisable to moisten it.

On 24 hour samples of urine, collected under toluene, analyses were conducted on the following constituents by familiar methods: calcium (5), magnesium (5), total phosphates (6), titratable acidity (7), total nitrogen (8), and ammonia nitrogen (9). All constituents except calcium and magnesium were determined on the fresh urine; for calcium and magnesium was used a solution obtained by a "wet ash" method.

From the 24 hour aggregate of feces was taken an aliquot for ashing in a muffle furnace, the resulting ash being dissolved in 5 per cent nitric acid and then analyzed for calcium, magnesium, and phosphorus by the methods just enumerated.

Once each week the animals were bled by cardiac puncture and analyses were conducted on the calcium, magnesium, and phosphorus of the serum by methods previously listed (4). All analyses were run in duplicate.

Results

Since the daily results on excretion of the three elements showed considerable fluctuation, the average daily excretion per week for each constituent was calculated. These figures were much more constant. The results for the preliminary period of 1 week, when all animals received the stock diet, precede the data during the experimental period. Of the two experimental animals, Dog 55 survived for a longer time than did Dog 56. In order to compare the effects of magnesium deprivation during the first few weeks on these two animals, as well as to compare the early with the late effects in Dog 55, the average daily values for the first 5 weeks, a length of time arbitrarily selected to accord with the survival period of Dog 56, were calculated for all dogs.

During the first 5 weeks on the control ration, Dog 57 showed an average daily excretion of 254.4 mg. of calcium, which represented its total output from both urine and feces (see Table I). Throughout the entire duration of the experiment, this animal had an average daily excretion of 248.3 mg. of calcium; hence it was losing calcium to approximately the same extent late in the experiment as earlier. In contrast to this, Dog 56 on the magnesium-low diet showed progressively diminishing excretion of calcium for the 5 weeks which it survived, so that the average daily excretion of total calcium for the 5 week period was only 144.0 mg., a figure much lower than that of the control animal. The other experimental animal, Dog 55, likewise showed a progressive lowering of calcium excretion during the first 5 weeks on the magnesium-deficient ration, the average daily excretion of total calcium during the time being 138.0 mg., which is strikingly similar to that of Dog 56 and much below the output of the control dog. Later Dog 55 excreted much larger amounts of calcium, the values for the average daily excretion for the 11th and 12th weeks being 446.0 mg. and 315.5 mg., which are far in excess of figures recorded for the control animal. Indeed, the elimination of this element increased to such a degree during the 6th to 12th weeks that the average daily excretion for the entire experimental period of 12 weeks amounted to 221.2 mg. of calcium, a figure which almost reaches that of the control animal.

Of particular interest is the metabolism of magnesium when the animal is deprived of this element. In the control Dog 57, the values 64.7 mg. and 72.6 mg. of magnesium, representing the average daily excretion over a 5 week period and a 13 week period respectively, indicate that when the animal body is supplied with adequate magnesium the excretion is practically constant. On the other hand, Dogs 55 and 56, receiving no magnesium, excreted much less during the 5 week period than did the control animal, their values being 33.3 mg. and 44.0 mg. per day on the average. During this time there is not, as in the case of calcium, a tendency to a progressive diminution; rather, the values remain consistently low. Even in the experimental animal that survived for 12 weeks, the average daily excretion of magnesium during the last 7 weeks differed little from that of the first 5 weeks. In the late stages of magnesium deficiency the constancy of magnesium excretion with values much below normal but steadily low is again in sharp contrast to progressively increasing elimination of calcium during the

TABLE I—*Total Average Daily Amounts of Calcium, Magnesium, and Phosphorus*
Recorded in Weekly Periods for Dogs 55 and 56, Deprived

Wk.	Total Ca			Total Mg			Total P			Total Ca:Mg	
	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
Preliminary	146.6	117.1	101.4	75.6	39.9	66.0	201.3	133.9	143.8	1.9	3.0
1	300.5	182.0	206.5	66.8	19.0	41.1	471.5	309.6	328.8	4.5	9.6
2	219.7	238.2	176.0	53.8	46.4	34.9	264.3	457.1	294.0	4.1	5.1
3		145.9	134.9		30.0	50.2		294.3	262.6		4.8
4	192.6	33.9	112.1	59.7	25.3	46.1	224.8	220.6	178.8	3.2	1.3
5	304.7	100.0	90.7	78.4	46.0	47.6	309.3	332.2	154.2	3.9	2.2
6	283.6	210.5		70.2	32.5		274.6	421.2		4.0	6.5
7	259.2	279.1		74.6	31.8		310.9	330.9		3.5	8.8
8	390.8	263.5		76.0	31.9		370.4	341.7		5.1	8.3
9	179.7	222.0		60.6	36.3		297.7	323.6		3.0	6.1
10	191.8	174.9		65.3	53.1		245.5	348.2		2.9	3.3
11	106.6	446.0		56.1	53.8		207.4	451.1		1.9	8.3
12	179.5	315.5		81.8	40.9		339.9	284.8		2.2	7.7
13	295.4			74.4			386.3			4.0	
ean, 5 wks.....	254.4	138.0	144.0	64.7	33.3	44.0	317.5	322.6	243.7	3.9	4.6
" all wks....	248.3	221.2	144.0	72.6	35.9	44.0	306.5	325.7	243.7	3.4	6.2
aximum.......	390.8	446.0	206.5	81.8	53.8	50.2	471.5	457.1	328.8	5.1	9.6
inimum........	106.6	33.9	90.7	53.8	19.0	34.9	207.4	220.6	154.2	1.9	1.3

That calcium and magnesium are excreted in the same proportion during the first 5 weeks by dogs on the magnesium-deficient diet, as by the dog on the control ration, is evident from the Ca:Mg ratios, since the values 4.6 and 3.3 for Dogs 55 and 56 respectively do not vary materially from 3.9, the value for the control Dog 57 which excreted absolutely greater amounts of both calcium and magnesium. In the later stages of magnesium deprivation, the increased elimination of calcium with magnesium remaining at the same low level produces a value that is almost double that of the control animal.

Excreted in Both Feces and Urine, Together with Blood Levels of These Constituents, of Magnesium, As Contrasted with Values for Control Dog 57

:	Total Ca:P			Total Mg:P			Blood Ca per 100 cc. serum			Blood Mg per 100 cc. serum			Blood P per 100 cc. serum		
Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56
							mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1.5	0.73	0.88	0.71	0.38	0.30	0.46	12.3	10.6	11.2	2.5	2.6	2.4	8.3	6.4	7.0
5.0	0.64	0.59	0.63	0.14	0.06	0.13	11.6	11.1	10.5	2.1	2.0	1.3	6.9	6.0	6.8
5.1	0.83	0.52	0.60	0.20	0.10	0.12	10.8	7.0	8.7	1.6	1.5	1.0	8.6	6.8	7.1
2.7		0.50	0.51		0.10	0.20		9.9	9.7		1.6	0.9		6.6	6.0
2.4	0.86	0.16	0.63	0.27	0.11	0.26	10.7	10.8	8.8	2.3	1.0	1.2	8.0	5.7	9.1
1.9	0.99	0.50	0.69	0.25	0.14	0.31	12.5	11.9		2.1	1.5		9.1	6.6	
	1.03	0.50		0.26	0.08		12.0	10.9		2.2	0.5		7.7	6.4	
	0.83	0.84		0.24	0.10		13.0	9.7		2.3	0.9		9.4	7.1	
	1.05	0.77		0.21	0.09		11.0	10.5		2.2	0.8		7.8	6.1	
	0.60	0.69		0.20	0.11		10.5	10.2		2.1	1.0		7.2	6.1	
	0.78	0.50		0.27	0.15		11.5	10.8		2.2	0.6		7.2	5.4	
	0.52	1.00		0.27	0.12			10.0			1.0			6.7	
	0.53	1.11		0.24	0.14		10.7	10.1		2.2	0.8		5.9	6.7	
	0.76			0.19											
3.3	0.83	0.41	0.59	0.22	0.10	0.18									
3.3	0.81	0.68	0.59	0.24	0.11	0.18	11.3	10.2	9.4	2.1	1.1	1.1	7.8	6.4	7.2
5.1	1.05	1.11	0.69	0.27	0.15	0.31	13.0	11.9	10.5	2.3	2.0	1.3	9.4	7.1	9.1
1.9	0.52	0.16	0.51	0.14	0.06	0.12	10.5	7.0	8.7	1.6	0.5	0.9	5.9	5.4	6.0

As is not entirely unexpected, values for phosphorus excretion in the two dogs on the magnesium-deficient ration show considerable difference. In one, the tendency is to a decreased output; in

the other to little or no change. This behavior of phosphorus excretion, together with the alterations in calcium and magnesium, is reflected in the Ca:P and Mg:P ratios.

Whether these changes in the total output of the three elements are brought about by the renal pathway, the intestinal channel, or both, are propositions to be demonstrated by recourse to specific data on these points. In the urine (Table II) the control Dog 57 excreted calcium over a period of 13 weeks at an average daily amount of 13.3 mg., which was not materially different from that of the first 5 weeks. In contrast to this figure, Dog 55 showed a slightly higher elimination of calcium in the urine, as is evident from the figures 16.1 and 17.4 mg., representing respectively the average daily output for the first 5 weeks and for the entire period on the magnesium-deficient ration. Still higher was the level of excretion of Dog 56, with a value for its 5 week survival period of 23.8 mg. per day. It should be noted, therefore, that the urine did not participate in the total calcium retention, already mentioned as characteristic of the early stages of magnesium deprivation, since the urinary excretion of this element in both of the experimental animals was definitely higher than that of the control. Furthermore, the renal channel is not responsible for the increased total calcium output seen in the late stages of magnesium deprivation since Dog 55 showed an average daily output of calcium in the urine for the entire period which was remarkably close to that of the first 5 weeks.

Unlike calcium, the excretion of magnesium in the urine was definitely diminished during the first 5 weeks on the diet, the average daily output of Dogs 55 and 56 being 20.1 mg. alike, as contrasted with a value of 38.8 mg. for the control animal, Dog 57. Nor did this diminution in excretion of magnesium undergo change during the late stages of the deficiency, since Dog 55 registered an average daily output of 22.0 mg. for the entire experimental period of 12 weeks. The slightly increased excretion of calcium and the lessened excretion of magnesium in the urine by the experimental animals, as contrasted with the control dog, is reflected in the Ca:Mg ratio which for Dog 55 was twice, and for Dog 56 three times as great as that for the control animal, Dog 57.

As in total phosphorus excretion, so in urinary phosphorus there is divergence between the two experimental animals. Curiously,

TABLE II

Average Daily Absolute Amounts of Calcium, Magnesium, and Phosphorus Excreted in Urine, Recorded in Weekly Periods for Dogs 55 and 56, Deprived of Magnesium, As Contrasted with Values for Control Dog 57

Wk.	Calcium			Magnesium			Phosphorus			Ca:Mg			Ca:P			Mg:P		
	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*									
Preliminary	11.9	35.6	10.1	22.7	13.6	29.2	117.3	69.2	96.8	0.52	2.62	0.35	0.10	0.51	0.11	0.19	0.20	0.30
1	8.7	15.0	50.0	27.6	12.1	17.6	256.6	251.6	232.0	0.31	1.24	2.84	0.03	0.06	0.21	0.11	0.05	0.08
2	10.8	22.8	46.5	26.3	17.6	19.6	190.9	347.9	175.6	0.41	1.29	2.36	0.06	0.07	0.26	0.14	0.05	0.11
3	24.8	16.0	7.5	83.6	18.5	29.2	142.7	255.5	221.1	0.30	0.97	0.26	0.17	0.06	0.03	0.59	0.07	0.13
4	15.2	6.4	5.8	33.9	15.3	18.1	122.2	191.7	123.0	0.45	0.42	0.32	0.12	0.03	0.05	0.28	0.08	0.15
5	6.0	20.5	9.3	22.4	37.2	16.1	91.1	273.6	94.8	0.26	0.55	0.58	0.07	0.07	0.10	0.25	0.14	0.17
6	8.8	11.5		29.9	21.9		109.3	312.3		0.29	0.53		0.08	0.04		0.27	0.07	
7	10.7	10.1		21.9	10.8		152.1	263.7		0.49	0.94		0.07	0.04		0.14	0.04	
8	22.7	7.5		21.2	21.0		204.7	243.4		1.07	0.36		0.11	0.03		0.10	0.09	
9	15.8	5.6		18.1	22.5		181.6	245.5		0.87	0.25		0.09	0.02		0.10	0.09	
10	6.0	11.9		16.0	44.3		129.3	297.5		0.37	0.27		0.05	0.04		0.12	0.15	
11	11.0	10.0		25.5	33.0		147.9	282.9		0.43	0.30		0.07	0.04		0.17	0.12	
12	26.3	83.8		45.9	25.9		256.0	212.0		0.57	3.23		0.10	0.39		0.18	0.12	
13	6.3	4.8		20.0	6.2		185.3	41.3		0.32	0.77		0.03	0.11		0.11	0.15	
Mean, 5 wks.......	13.1	16.1	23.8	39.0	20.1	20.1	160.7	264.1	169.3	0.35	0.80	1.27	0.09	0.06	0.13	0.27	0.08	0.13
" all wks.....	13.3	17.4	23.8	30.2	22.0	20.1	166.9	247.6	169.3	0.47	0.85	1.27	0.08	0.08	0.13	0.20	0.09	0.13
Maximum.........	26.3	83.8	49.9	83.6	44.3	29.2	256.6	347.9	232.0	1.07	3.23	2.84	0.17	0.39	0.26	0.59	0.15	0.17
Minimum.........	6.0	4.8	5.8	16.0	6.2	16.1	91.1	41.3	94.8	0.26	0.25	0.26	0.03	0.02	0.03	0.10	0.04	0.08

the dog with total phosphorus excretion on a par with the control values shows a higher urinary excretion of phosphorus; while the dog with total phosphorus excretion below the control figures shows urinary phosphorus excretion comparable to that of the control animal. With such dissimilarity in the urinary phosphorus excretion of the two experimental animals, it is manifest that their Ca:P and Mg:P ratios of the urine will be unlike.

From the data on the urinary excretion of calcium, it is apparent that it does not participate in the retention of total calcium during the early stages of magnesium deprivation. On the other hand, the diminished excretion of magnesium through the urinary pathway does contribute to the lessened total elimination of this element which occurs in magnesium deficiency. If calcium retention is not due to changes in the output through the urinary channel, it must be due to lessened excretion through the intestinal tract. It is, moreover, not impossible that lessened fecal excretion may contribute with diminished urinary excretion to the retention of magnesium.

In both animals restricted to the magnesium-deficient ration, calcium excretion in the feces is definitely lowered during the early stages; values of 123.8 mg. and 120.2 mg. per day for Dogs 55 and 56 respectively during the first 5 weeks fall much below 244.2 mg. per day for the control animal (Table III). As with the excretion of total calcium, the elimination of calcium by the alimentary route is definitely increased during the later stages of magnesium deficiency and approaches but never reaches the level of excretion by the control animal, since Dog 55 showed over a period of 12 weeks an average daily excretion of fecal calcium amounting to 199.2 mg., which was definitely higher than its figure of 123.8 mg. per day for the first 5 weeks but lower than the value 218.2 mg. per day recorded for the control dog for 13 weeks.

In accord with diminished total excretion and lessened urinary excretion of magnesium is the definite reduction in output of magnesium in the feces of the experimental animals. During the first 5 weeks on the experimental diet, Dogs 55 and 56 eliminated 13.2 mg. and 23.9 mg. per day respectively by the intestinal pathway, as contrasted with the value 37.1 mg. per day for the control animal. Nor did the length of the experiment have any influence on the level of magnesium excretion in the feces since Dog 55

TABLE III

Average Daily Absolute Amounts of Calcium, Magnesium, and Phosphorus Excreted in Feces, Recorded in Weekly Periods for Dogs 55 and 56, Deprived of Magnesium, As Contrasted with Values for Control Dog 57

Wk.	Calcium			Magnesium			Phosphorus			Ca:Mg			Ca:P			Mg:P		
	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*									
Preliminary	134.7	81.5	91.3	52.9	26.3	36.8	84.0	64.7	47.0	2.5	3.1	2.5	1.6	1.3	1.9	0.63	0.41	0.78
1	291.8	167.0	156.6	39.2	6.9	23.6	214.9	58.0	96.8	7.6	24.2	6.7	1.4	2.9	1.6	0.18	0.12	0.24
2	208.9	215.4	129.5	27.5	28.8	15.2	73.4	109.2	118.4	7.6	7.5	8.5	2.9	2.0	1.1	0.38	0.26	0.13
3		129.9	127.4		11.5	21.1		38.8	41.5		11.3	6.0		3.4	3.1		0.30	0.51
4	177.4	27.5	106.3	25.8	10.0	28.0	102.6	28.9	55.8	6.9	2.8	3.8	1.7	1.0	1.9	0.25	0.35	0.50
5	298.7	79.5	81.4	56.0	8.7	31.5	218.2	58.6	59.4	5.3	9.1	2.6	1.4	1.4	1.4	0.26	0.15	0.53
6	274.8	199.0		40.4	10.7		165.3	108.9		6.8	18.7		1.7	1.8		0.24	0.10	
7	248.5	269.0		52.7	21.1		158.8	67.2		4.7	12.8		1.6	4.0		0.33	0.31	
8	368.1	256.0		54.8	10.9		165.7	98.3		6.7	23.5		2.2	2.6		0.33	0.11	
9	163.9	216.4		42.5	13.9		116.1	78.1		3.9	15.8		1.4	2.8		0.37	0.18	
10	185.8	163.0		49.3	8.8		116.2	50.7		3.8	18.5		1.6	3.2		0.42	0.17	
11	95.6	436.0		30.5	20.8		59.5	168.2		3.1	20.9		1.6	2.6		0.51	0.12	
12	153.2	231.7		35.8	15.0		83.9	72.8		4.3	15.5		1.8	3.2		0.43	0.21	
13	289.1			54.4			201.0			5.3			1.4			0.27		
Mean, 5 wks.......	244.2	123.8	120.2	37.1	13.2	23.9	152.3	58.7	74.4	6.3	9.4	5.0	1.8	2.1	1.6	0.27	0.22	0.32
" all wks.....	218.2	199.2	120.2	42.4	13.9	23.9	139.6	78.1	74.4	5.2	14.3	5.0	1.6	2.6	1.6	0.30	0.18	0.32
Maximum.........	368.1	436.0	156.6	56.0	28.8	31.5	218.2	168.2	118.4	7.6	24.2	8.5	2.9	3.4	3.1	0.51	0.35	0.53
Minimum.........	95.6	27.5	81.4	25.8	6.9	15.2	59.5	28.9	41.5	3.1	2.8	2.6	1.4	1.0	1.1	0.18	0.10	0.13

showed a value of 13.9 mg. per day over a stretch of 12 weeks, an amount which was almost identical with that for the first 5 weeks.

Comparable to the diminished excretion of total calcium and total magnesium during the early stages of magnesium deprivation, the decreased excretion of fecal calcium and fecal magnesium is almost proportionate since the Ca:Mg ratios of the feces of these animals are not far from that of the control animal. The greater fecal loss of calcium in the late stages, however, yields a Ca:Mg ratio that is much higher than that of the control animal for the same period.

As the result of magnesium deprivation, the elimination of phosphorus in the feces was markedly diminished. Since fecal excretion of all three constituents is diminished in the early stages of magnesium deficiency, the Ca:P and Mg:P ratios in feces suffer no striking alteration during this time.

In the control animal, the excretion of calcium is so divided between the urine and feces that during the first 5 weeks on the control diet the animal had a urine calcium to feces calcium ratio of 0.05 and for the entire 13 weeks of 0.06 (Table IV). It has been mentioned that in the early stages of magnesium deprivation Dogs 55 and 56 showed a lowered excretion of total amount of calcium because of lessened output through the intestinal channel; hence the partition of calcium in the excretory paths undergoes a change in which the urine participates to a greater extent in the elimination of this element. This change is reflected in the urine calcium to feces calcium ratios of both animals, the values being 0.16 and 0.18 respectively. Even when the amount of calcium eliminated in the feces is increased in absolute amount during the late stages of the deficiency, the urine calcium to feces calcium ratio does not assume a normal value although it tends somewhat in this direction.

Just as calcium shows an altered distribution in its excretory channels, as the result of magnesium deficiency, so magnesium and phosphorus manifest an almost identical behavior. Although the absolute amounts of magnesium excreted by the experimental animals are decreased in both urine and feces, the proportion is such that relatively more magnesium passes out through the urinary pathway than through the intestinal canal. For the first 5 weeks, Dogs 55 and 56 had urine magnesium to feces magnesium ratios of 1.9 and 0.9 respectively, as contrasted with 0.8 of the control animal. In the later stages of magnesium deficiency this new partition remained unchanged for Dog 55.

Under the experimental conditions, phosphorus likewise passed from the body to a greater extent in the urine than in the feces, since Dogs 55 and 56 evidenced ratios of 5.1 and 2.6, in contrast to 1.4 for the control animal. Here again, the continued effects of magnesium deprivation did not bring the ratio to normal.

TABLE IV

Ratios Showing Distribution of Calcium, Magnesium, and Phosphorus between the Two Pathways of Excretion, Urine and Feces, Recorded in Weekly Periods for Dogs 55 and 56, Deprived of Magnesium, As Contrasted with Values for Control Dog 57

Wk.	$\frac{\text{Urine Ca}}{\text{Feces Ca}}$			$\frac{\text{Urine Mg}}{\text{Feces Mg}}$			$\frac{\text{Urine P}}{\text{Feces P}}$		
	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56
Preliminary	0.09	0.44	0.11	0.4	0.5	0.8	1.4	1.1	2.1
1	0.03	0.09	0.32	0.7	1.7	0.7	1.2	4.3	2.4
2	0.05	0.11	0.36	1.0	0.6	1.3	2.6	3.2	1.5
3		0.12	0.06		1.6	1.4		6.6	5.3
4	0.09	0.23	0.05	1.3	1.5	0.6	1.2	6.6	2.2
5	0.02	0.26	0.11	0.4	4.3	0.5	0.4	4.7	1.6
6	0.03	0.06		0.7	2.1		0.7	2.9	
7	0.04	0.04		0.4	0.5		1.0	3.9	
8	0.06	0.03		0.4	1.9		1.2	2.5	
9	0.10	0.03		0.4	1.6		1.6	3.2	
10	0.03	0.07		0.3	5.0		1.1	5.9	
11	0.11	0.02		0.8	1.6		2.5	1.7	
12	0.17	0.36		1.3	1.7		3.1	2.9	
13	0.02			0.4			0.9		
Mean, 5 wks......	0.05	0.16	0.18	0.8	2.0	0.9	1.4	5.1	2.6
" all wks.....	0.06	0.12	0.18	0.7	2.0	0.9	1.4	4.0	2.6
Maximum........	0.17	0.36	0.36	1.3	5.0	1.4	3.1	6.6	5.3
Minimum........	0.02	0.02	0.05	0.3	0.5	0.5	0.4	1.7	1.5

In order that the changes in the calcium, phosphorus, and magnesium concentration of the blood serum might be studied concomitantly with the alterations in the metabolism of these elements, determinations on the blood were made weekly with the results seen in Table I. In confirmation of previous studies in this

TABLE V

Total Average Daily Amounts of Nitrogen, Ammonia, and Acid Excreted in Urine, Recorded in Weekly Periods for Dogs 55 and 56, Deprived of Magnesium, As Contrasted with Values for Control Dog 57

Wk.	Weight			Volume of urine			Total N			N per kilo of body weight			NH_3-N			Acidity, cc. 0.1 N		
	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56	Dog 57	Dog 55	Dog 56
	kg.	*kg.*	*kg.*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*cc.*	*cc.*	*cc.*
Preliminary	2.8	3.0	2.8	201.0	197.0	186.0	1.5	0.7	1.4	0.54	0.25	0.50	56.8	109.2	86.6	34.7	37.1	24.7
1	3.3	3.0	3.1	199.9	372.0	182.5	2.0	0.9	1.7	0.61	0.31	0.53	61.7	58.7	55.1	63.5	31.5	49.6
2	3.8	3.6	3.6	132.3	331.0	143.5	1.2	1.7	1.2	0.31	0.47	0.34	105.2	53.1	89.7	15.2	54.3	40.2
3	3.3	4.0	3.6	345.3	394.2	213.0	1.7	1.6	1.5	0.53	0.40	0.43	47.6	82.0	111.9	47.3	79.9	45.6
4	3.4	3.9	3.6	284.0	193.0	128.0	1.3	2.0	1.2	0.38	0.51	0.34	74.1	48.2	99.7	30.7	88.3	29.7
5	3.5	3.9	3.3	144.0	262.7	133.0	0.9	1.7	0.8	0.27	0.43	0.26	45.2	32.2	51.4	25.6	98.1	28.1
6	3.8	4.1		221.0	309.0		1.2	1.5		0.31	0.37		70.1	26.0		37.1	71.1	
7	4.0	4.3		126.0	250.6		1.0	1.7		0.25	0.41		97.5	25.2		38.1	63.1	
8	4.7	4.2		150.0	192.0		1.6	2.1		0.33	0.50		116.7	46.6		39.3	72.0	
9	5.0	4.2		189.0	220.0		1.1	2.3		0.23	0.56		112.9	57.3		39.8	75.3	
10	5.3	4.1		139.0	220.0		1.4	2.0		0.26	0.49		105.0	92.4		28.6	98.8	
11	5.3	3.9		262.2	304.0		1.4	2.5		0.27	0.64		101.9	92.7		40.2	81.4	
12	5.4	3.9		490.4	195.7		1.5	2.8		0.27	0.73		126.1	86.6		60.9	121.8	
13	5.3	3.8		240.0	57.0		1.2	1.1		0.23	0.29		92.3	30.9		34.8	38.1	
Mean, 5 wks.......							1.4	1.6	1.3	0.42	0.43	0.38	66.8	54.8	81.6	36.4	70.4	38.6
" all wks.....	4.3	3.9	3.4	224.7	254.0	160.0	1.3	1.9	1.3	0.33	0.47	0.38	89.0	56.2	81.6	38.5	74.9	38.6
Maximum.........	5.4	4.3	3.6	490.4	394.2	213.0	2.0	2.8	1.7	0.61	0.73	0.53	126.1	92.7	111.9	63.5	121.8	49.6
Minimum.........	3.3	3.0	3.1	126.0	57.0	128.0	0.9	0.9	0.8	0.23	0.29	0.26	45.2	25.2	51.4	15.2	31.5	28.1

series (4), it is evident that magnesium is the only constituent of the three which undergoes change, and that this element undergoes diminution early in the deficiency. Despite the characteristic changes in the excretion of absolute amounts of calcium, magnesium, and phosphorus, as well as the alteration in the proportion of their passing through the two pathways, the concentrations of calcium and phosphorus in the blood give no indication, as is not unexpected, of changes in their metabolism.

As was pointed out by Kruse, Orent, and McCollum (4), deficiency diseases manifest their effects both locally and constitutionally upon the body. Magnesium deficiency is no exception; the local effect is a distinct form of tetany, the constitutional effect is nutritive failure. From the standpoint of differentiation of magnesium tetany from all other forms, the determination of ammonia production and acidity of urine assumes significance; while from the standpoint of nutritive failure, the urinary excretion of nitrogen is not without value as evidence. In Dog 56, which died early from convulsions, the titratable acidity and ammonia excretion were almost identical with those of the control dog (Table V). Dog 55 throughout the period of magnesium deficiency, on the other hand, showed a slightly diminished excretion of ammonia and an increased titratable acidity which in the amounts determined indicated neither an alkalosis nor an acidosis. As regards nitrogen metabolism, Dog 56 with a short survival period undoubtedly succumbed to the local disturbances in the nervous system before the full development of nutritive failure. Dog 55, however, showed an ever increasing nitrogen metabolism throughout the deficiency with values far in excess of those of the control animal. For example, from the 9th to 12th weeks, this animal showed an average daily elimination of 2.3, 2.0, 2.5, and 2.8 gm. of nitrogen respectively, while the control animal during the same period excreted not more than 1.5 gm. per day. This increased elimination of nitrogen is also borne out when the values are calculated on the basis of excretion of nitrogen per kilo of body weight (Table V).

DISCUSSION

While magnesium is tenaciously held throughout the entire survival period by animals restricted to the magnesium-deficient ration, calcium is likewise retained to an inordinate degree during the first 5 weeks of the deficiency, after which it is excreted in progressively increasing amounts. This sudden reversal of calcium metabolism is suggestive of two forces at work, in which the one operating towards calcium conservation yields soon to the other inducing calcium elimination. It is therefore with valid reason that in the endeavor to ascertain the responsible forces we demarcate the metabolic processes of calcium into two separate stages, the one retention up to the 6th week, the other loss thereafter.

Of these two aspects of calcium metabolism, the stage of retention is the more singular. That an animal deprived of magnesium should attempt to husband what magnesium it possesses in its body is an expected event, but that calcium, abundantly supplied in the ration, should likewise be retained to an unusual degree is not so reasonable. Here necessity is not a controlling factor; another agency must be in operation. Under these circumstances it should not be overlooked that a physiological relationship has been postulated between calcium and magnesium.

Towards each other, calcium and magnesium are said to exert a mutually antagonistic action. Inasmuch as the origin of this idea and the nature of its supporting evidence from such sources as nerve irritability and bone composition are matters of record which have been adequately reviewed (10), we shall not dilate upon them; instead we shall confine our comment to the metabolic changes bearing upon the point. It is true that the ability of increased calcium intake to heighten excretion of magnesium, although asserted on one occasion (11), has not been generally admitted (12–16). But the case is quite different for the converse. When magnesium salts are injected into the animal body, calcium excretion is stimulated usually through the urinary channel (15, 17–23); if the magnesium salts are ingested, the same effect is said to prevail (12, 24–28), although this latter evidence has been challenged in a few instances and in a none too convincing manner (14, 16, 29, 30). In keeping with the increased elimination of calcium, a lowered concentration of calcium in the blood has been found following magnesium administration (18, 22, 27, 30–32), but here again is lack of unanimity (16, 23, 26, 33). If all these data were submitted to a poll, if equal weight with no disqualifications was given to each, the majority would indicate that calcium administration is without effect on magnesium metabolism; but that magnesium administration may be presumed to exert a real influence on calcium metabolism. For present purposes, it is sufficient to infer from the latter and more pertinent point that magnesium does show a physiological relationship in the control of calcium metabolism.

Such a casual consideration, while favorable to the view that magnesium wields control over calcium metabolism, does not do full justice to it, since critical analysis reduces the force of much of the opposing data. So diverse have been the technical details involved in the investigations, the wonder is that there has been a semblance of similarity in the results. It becomes apparent that such matters as the length of the experiment, the amount of magnesium administered, and the method of its introduction into the body account for not a few discrepancies and contradictions. For example, some experiments involved oral administration without due allowance for the uncertain and often disturbing behavior of magnesium salts in the intestine; other experiments suffered from too early termination. Even more confounding has been the neglect in many instances to take cognizance of the influence which the less obvious but none the less important factors such as the level of calcium intake, the level of phosphorus intake (23, 24, 26, 34–37), and the nature of the anion associated with magnesium (22, 27), have recently been shown to exert on the calcium-magnesium relationship. The implication of phosphorus is not unexpected. With the elucidation of the pathogenesis of experimental rickets came an appreciation of the relationship between calcium and phosphorus, both absolutely and relatively, in the effects upon blood chemistry, metabolism, and bone composition. Now, quite an imposing list of experimental results attests to the fact that not only does phosphorus control calcium, but also if the level of phosphorus intake is sufficiently high it may suppress the antagonistic effect of magnesium on calcium. At a low plane of intake phosphorus is inoperative. Finally, it has been asserted that, due to the power of the anion in union with magnesium, retention or elimination of calcium may be determined by selection of appropriate magnesium salts. When the experiments with outstanding complicating factors of these kinds are removed from consideration, it is not difficult to reach the conclusion that fundamentally magnesium and calcium exhibit a physiological relationship whereby magnesium, as truly as phosphorus, determines the metabolism of calcium.

It is not enough to reveal the shortcomings and complicating factors of experiments that would negate the existence of this relationship; it is desirable to present fresh evidence of a positive nature, particularly evidence that would carry an explanation. Unquestionably the idea of a relationship between magnesium and calcium would take on new credence if the mechanism could be visualized. To speak of this relationship as physiological is scarcely satisfying; to reduce it to a chemical basis is more promising. To this end physicochemical studies on the behavior of magnesium and calcium salts *in vitro* may be of profit in their revelations that magnesium and calcium are capable of forming complex salts with such substances as amino acids; that the greater stability of the magnesium complex makes likely the possibility of displacement of calcium by magnesium in such complexes; and that increased solubility of calcium salts in the presence of magnesium compounds is due in part to formation of a soluble complex (38). If we may assume the validity of the observations that introduction of magnesium into the body leads to an increase in ultrafiltrable serum calcium (31, 39), a decrease in ultrafiltrable serum phosphate (39), and increased excretion of calcium, the physicochemical findings not only support these assumptions but also explain them on a rational basis. It is not difficult to picture that by displacement magnesium releases ionized calcium in a form ready for excretion. True the matter lacks the touch of finality, but the intimation is strong that magnesium, as truly as phosphorus, is a governing factor in the regulation of calcium metabolism.

In the foregoing instances cited from the literature, the antagonism operates under a circumstance in which magnesium is added in more than an ordinary amount. That the antagonism may likewise prevail in a condition where magnesium is supplied in insufficient amount to the body is apparent from the evidence in the present study, where absence of magnesium from the ingested ration has led to retention of calcium. The physiological antagonism of magnesium and calcium would seem to be, accordingly, the governing force which produced calcium retention during the first 5 weeks of magnesium deprivation.

This antagonistic process tending to calcium retention is soon completely overcome and turned in the opposite direction. From the 6th week on the animal excretes calcium in ever increasing amounts. It seems more than a coincidence that the 6th week in the survival period likewise usually marks the onset of nutritive failure as judged by alteration in the concentration of certain lipids in the blood. At this time bodily disintegration attendant upon nutritive failure sets in and calcium is among the substances lost during the breakdown. Just as in fasting, so in the late stages of magnesium deficiency—both conditions inducing inanition—profound tissue destruction and increased excretion of calcium predominate. Waiving all minor differences between the rate of excretion in the two cases, we may note that, taken by and large, both conditions are accompanied by loss of calcium. It is not improbable, therefore, that nutritive failure is the circumstance which intervenes to destroy the tendency to calcium retention early in magnesium deprivation.

In direct contrast to the reversal of calcium excretion, the constancy of magnesium elimination, even in the face of nutritive failure, is a noteworthy feature in magnesium deficiency. Next to its high content in bone, magnesium is distributed to a great extent in muscle and such cellular organs as undergo disintegration in fasting, whereupon magnesium excretion becomes heightened. Despite the fact that a similar breakdown of tissues probably occurs in magnesium deficiency, the animal does not excrete magnesium upon its release but retains it tenaciously throughout the survival period. The need for magnesium apparently constitutes a force which overcomes the tendency to increased magnesium excretion.

Another aspect of nutritive failure, as it is manifested in magnesium deficiency, is open to interpretation from the data in hand. It has been mentioned that both magnesium deficiency and fasting show nutritive failure in common, but in certain respects the behavior of blood lipids is different. Regardless of the intermediate changes, about which there is little concordance of evidence, much less of opinion (40, 41), impending death from fasting with its accentuated protein metabolism is often reflected by the so called premortal rise of nitrogen excretion in the urine. Indeed, fasting animals show ever increasing urinary nitrogen excretion, with or without premortal rise, as a characteristic feature. In magnesium deficiency, Kruse, Orent, and McCollum asserted that a terminal rise in non-protein nitrogen appeared in the blood (4). This change, together with a marked disturbance in blood lipids was interpreted as a failure in fat metabolism, not necessarily due to depletion of body fat, which ultimately involved protein metabolism. The validity of this hypothesis should receive some support from the behavior of urinary nitrogen excretion. In the present study, the one experimental animal which survived for a sufficient time to develop nutritive failure showed a steadily rising nitrogen excretion which reached its maximum just before death.

Finally, in a previous paper we asserted on the basis of blood findings (4) that alkalosis, which has been cited in some quarters as the precipitating factor in many if not all forms of tetany (42), was not present at any time in magnesium tetany. The urinary findings, revealed by data on ammonia and acid excretion, bear out this contention, and confirm, if indeed further evidence is needed, the distinctive character of magnesium tetany.

SUMMARY

During the early stages of magnesium deficiency, the course of calcium metabolism is definitely to progressively increasing retention; thereafter it is to progressively increasing elimination. Magnesium excretion, more constant not only in its course but also in its magnitude, is decidedly diminished throughout the entire survival period, with no change in the level even in the late stages. Contrariwise the elimination of phosphorus is inconstant; this element may or may not be retained to an unusual degree, but it is never excessively excreted.

Of the two channels involved in the excretion of calcium, magnesium, and phosphorus, the intestine predominates over the kidney in retaining these elements in the body during the first period of magnesium deficiency. Indeed magnesium alone shows diminished output in the urine. The retention of calcium and phosphorus is entirely, that of magnesium largely, due to their markedly diminished output in the feces; consequently their use of the pathways of excretion is altered, with the urine participating to a relatively greater degree than is usual. The increased excretion of total calcium in the late stages of the deficiency is due to a greater excretion of this element in the feces.

It is suggested that the calcium retention in the early stages of the deficiency is due to the antagonism known to exist between calcium and magnesium. The breakdown in calcium retention is due, in all probability, to nutritive failure. The progressively increasing excretion of nitrogen, taken in conjunction with previously reported terminal increase in non-protein nitrogen values of the blood, is likewise attributed to the failure of nutritive processes.

The urinary data on acid and ammonia excretion in magnesium deficiency show absence of alkalosis, and thereby lend support to the blood findings which previously identified magnesium tetany as a separate and distinct entity.

BIBLIOGRAPHY

1. Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, **96**, 519 (1932).
2. Kruse, H. D., Schmidt, M. M., and McCollum, E. V., *Am. J. Physiol.*, **105**, 635 (1933).
3. Orent, E. R., Kruse, H. D., and McCollum, E. V., *Am. J. Physiol.*, **101**, 454 (1932).
4. Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, **100**, 603 (1933).
5. Tisdall, F. F., and Kramer, B., *J. Biol. Chem.*, **48**, 1 (1921).
6. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
7. Folin, O., *Am. J. Physiol.*, **9**, 265 (1903).
8. Folin, O., and Denis, W., *J. Biol. Chem.*, **26**, 473 (1916).
9. Folin, O., and Macallum, A. B., *J. Biol. Chem.*, **11**, 523 (1912).
10. Elmslie, W. P., and Steenbock, H., *J. Biol. Chem.*, **82**, 611 (1929).
11. Mendel, L. B., and Benedict, S. R., *Am. J. Physiol.*, **25**, 23 (1909–10).
12. Malcolm, J., *J. Physiol.*, **32**, 183 (1905).
13. Givens, M. H., *J. Biol. Chem.*, **34**, 119 (1918).
14. Underhill, F. P., Honeij, J. A., and Bogert, L. J., *J. Exp. Med.*, **32**, 65 (1920).
15. Whelan, M., *J. Biol. Chem.*, **63**, 585 (1925).
16. Watchorn, E., *J. Hyg.*, **32**, 156 (1932).
17. Mendel, L. B., and Benedict, S. R., *Am. J. Physiol.*, **25**, 1 (1909–10).
18. Stransky, E., *Arch. exp. Path. u. Pharmakol.*, **78**, 122 (1915).
19. Jacoby, M., *Biochem. Z.*, **74**, 131 (1916).
20. Schiff, E., *Jahrb. Kinderh.*, **91**, 43 (1920).
21. Magnus-Levy, A., *Z. klin. Med.*, **107**, 671 (1928).

22. Pribyl, E., *Compt. rend. Soc. biol.*, **102**, 258 (1929).
23. Barbour, H. G., and Winter, J. E., *J. Pharmacol. and Exp. Therap.*, **43**, 607 (1931).
24. Hart, E. B., and Steenbock, H., *J. Biol. Chem.*, **14**, 75 (1913).
25. Bogert, L. J., and McKittrick, E. J., *J. Biol. Chem.*, **54**, 363 (1922).
26. Palmer, L. S., Eckles, C. H., and Schutte, D. J., *Proc. Soc. Exp. Biol. and Med.*, **26**, 58 (1928).
27. Bečka, J., *Z. ges. exp. Med.*, **67**, 253 (1929).
28. Roche, A., *Bull. Soc. chim. biol.*, **14**, 634 (1932).
29. Huffman, C. F., Robinson, C. S., Winter, O. B., and Larson, R. E., *J. Nutrition*, **2**, 471 (1930).
30. Watchorn, E., *Brit. J. Exp. Path.*, **7**, 120 (1926).
31. Richter-Quittner, M., *Z. ges. exp. Med.*, **45**, 479 (1925).
32. Brookfield, R. W., *Biochem. J.*, **27**, 173 (1933).
33. Lang, K., *Z. klin. Med.*, **122**, 206 (1932).
34. Kramer, B., Shelling, D. H., and Orent, E. R., *Bull. Johns Hopkins Hosp.*, **41**, 426 (1927).
35. Shelling, D. H., Kramer, B., and Orent, E. R., *J. Biol. Chem.*, **77**, 157 (1928).
36. Haag, J. R., and Palmer, L. S., *J. Biol. Chem.*, **76**, 367 (1928).
37. Carswell, H. E., and Winter, J. E., *J. Biol. Chem.*, **93**, 411 (1931).
38. Zörkendörfer, W., *Biochem. Z.*, **221**, 33 (1930).
39. Scholtz, H. G., *Biochem. Z.*, **231**, 135 (1931).
40. Voit, E., *Z. Biol.*, **41**, 550 (1901).
41. Gamble, J. L., Ross, G. S., and Tisdall, F. F., *J. Biol. Chem.*, **57**, 633 (1923).
42. Stepp, W., and György, P., Avitaminosen und verwandte Krankheitszustände, Berlin (1927).

STUDIES ON MAGNESIUM DEFICIENCY IN ANIMALS

VI. CHEMICAL CHANGES IN THE BONE, WITH ASSOCIATED BLOOD CHANGES, RESULTING FROM MAGNESIUM DEPRIVATION

By ELSA R. ORENT,* H. D. KRUSE, and E. V. McCOLLUM

(*From the Biochemical Laboratory, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore*)

(Received for publication, June 26, 1934)

After the experimental production of magnesium deficiency, the investigation of its effects on the animal body embraced in succession the tetanic disturbances of the nervous system, the alteration in the chemical constituents of the blood, and the changes in the metabolism of calcium, magnesium, and phosphorus (1–5). In the preceding communication it was indicated that, when the magnesium intake is almost infinitesimal, the magnesium excretion is decidedly diminished throughout the entire survival period. Calcium metabolism too, in the course of magnesium deprivation, is not exempt from alteration; for a continuously increasing retention of calcium, induced at the outset, gives way later with a complete reversal to a continuously increasing elimination. As an explanation for the unnatural behavior of calcium metabolism, it was suggested that the retention in the early stages is due to the physiological control which magnesium is reputed to exert over calcium, while the later excessive loss is due in all likelihood to nutritive failure.

The very nature of these metabolic changes prompted, as the next step in the study of magnesium deficiency, an examination of the effects on bone. Regardless of the credible view that magnesium activates phosphatase (6) which in turn affects bone formation and thus involves magnesium in the regulation of ossification (7), we became concerned in the osseous system primarily on the more general ground of the close relationship which the metabolism of calcium, magnesium, and phosphorus bears to its structure. In the present investigation we sought to determine whether magnesium deprivation with its singular and profound changes in the elimination of these three elements would actually cause, what theory suggested, an altered composition of the bone, and whether the control of magnesium over calcium, seen in the metabolism studies, might be further demonstrated in the bone. Besides we were desirous of having the chemical data of the blood, which in dogs had comprised an unaltered calcium and phosphorus but a prompt and considerable lowering of the magnesium concentration, not only confirmed in the rat but also correlated with any concomitant changes in the bone composition.

---

* National Research Fellow in Biological Sciences (Biochemistry) 1930–32.

TABLE I

Content of Calcium, Magnesium, and Phosphorus in Control and Low Magnesium Rations

Ration No.	100 parts of diet			Ca:P	Ca:Mg
	Calcium	Magnesium	Phosphorus		
11 (control)	0.60	0.054	0.38	1.55	11.1
10 (low Mg)	0.60	0.00018	0.38	1.55	3336.5

Technique and Methods

Young rats weighing from 35 to 45 gm. were, after weaning, restricted to a diet deficient in magnesium but otherwise adequate for the period of experimentation; while another group of rats of similar age and weight was placed on the control diet containing ample magnesium. The composition of these diets has already been described (1). The content of calcium, phosphorus, and magnesium, as well as the ratio of these elements, in the two diets is presented in Table I.

Because repeated determinations on the same animal were impossible, the analyses of blood and bone in both groups were conducted at definite and regular periods of time on animals which had not previously been examined. In order that a basis might be afforded for determining the effects due to age in the case of the control animals, and the effects due to diet in the case of the experimental animals, a third group of animals was sacrificed at once after weaning. Thereafter animals from the first two groups were sacrificed at 5 day intervals over a period of 33 days, the control animals in each instance furnishing data which showed the changes referable to age, and which served as a basis for contrast with those of the experimental animals. In all instances of animals on the control diet and in most instances on the experimental diet, both blood and bone were analyzed in the same group of animals. Where these corresponding values are available they are so designated in Table III. In a limited number of instances of animals on the experimental diet the blood alone was analyzed. Usually by design but sometimes by chance, the animals restricted to the magnesium-deficient ration were sacrificed, among other times, during the height of vasodilatation or during the first stage of induced convulsions; in either case such a circumstance is designated in the table (Tables III and V). Still other experimental animals were sacrificed without inducing convulsions. This procedure revealed the effects of convulsions on blood and bone composition.

Since a rat of the age used in this work furnishes bone and blood samples insufficient for analyses, particularly in duplicate, a number of rats ranging from two to nine were sacrificed at each stage and their blood and bones were pooled respectively for the determinations. The number of rats which were combined for each analysis and the sex distribution are also enumerated in the tables (Tables II to V and Tables IV and V respectively). The values derived from such a combination of animals are really equivalent to the arithmetical mean of the figures obtained by separate and individual analyses for the same animals. The blood was obtained from animals sacrificed without anesthesia, the samples from all rats in each group being collected in a single tube. The methods of analysis for calcium, magnesium, and phosphorus in the blood have already been recorded (4).

In preparing the bones for analysis, we adopted a procedure which combined a satisfactory degree of expediency and exactness. Immediately after death the hind extremities of the animals were severed from the body. After the integument had been stripped away, the extremities were immersed in boiling distilled water for 3 minutes. The muscle was removed and the femora, tibiæ, and fibulæ were taken for analysis. The bones were washed with distilled water to remove any adherent strands of tissue; they were dried with filter paper; and they were combined according to the same grouping of animals from which blood had been pooled. After the bones were weighed and dried in an oven at 100–110° until constant in weight, they were incinerated in a muffle furnace at 440–450° in platinum dishes until a pure white ash was obtained. 25 mg. of the bone ash were weighed and transferred to a dry 25 cc. volumetric flask. 5 cc. of 1 N hydrochloric acid were added, and when the ash was entirely dissolved, the volume of the solution was brought to 25 cc. with distilled water. Calcium, magnesium, and phosphorus were determined by duplicate analyses in aliquot portions, the methods used being those already cited (4). While we are aware that extraction of bones by a lipid solvent prior to ashing is listed as the preferred method, because it is purported to reduce the error from organic phosphorus (8) and to yield more accurate results for percentage of ash in the bone by excluding the variable fat content (9), neither objection is likely to exert much influence on the absolute amounts of calcium and magnesium in the bone. On the other hand, the preliminary treatment by boiling rendered it inadvisable that we use calculations upon fresh bone, as was Hammett's practise (10). We have, therefore, based our determinations upon dry bone.

TABLE II

Calcium, Magnesium, and Phosphorus Content of Serum from Young Rats Which Had Received the Control Ration for Various Periods of Time

Initial age	Final age	Days on control diet	No. of animals	Mg. per 100 cc. serum			Ca:Mg
				Calcium	Magnesium	Phosphorus	
days	*days*						
25	25	0	9	11.4	2.9	11.2	3.8
25	30	5	5	9.4	3.1	13.9	3.0
25	35	10	4	9.2	3.2	12.5	2.9
25	40	15	4	9.2	2.8	11.2	3.2
25	45	20	4	10.6	3.3	11.7	3.2
25	50	25	4	10.7	3.1	11.7	3.4
25	55	30	4	10.2	3.3	10.8	3.0
25	58	33	4	10.5	3.2	10.1	3.2

Results

For purposes of reestablishing the normal figures for serum calcium, magnesium, and phosphorus in young rats, of judging the suitability of the control diet with respect to these factors, and of providing a basis of ascertaining the extent of chemical changes in the blood of the experimental animals, the results of the blood analyses conducted on the control animals are recorded in Table II. In contrast, the behavior of serum calcium, magnesium, and phosphorus during magnesium deprivation is revealed in Table III. With rats, as with dogs, restriction to a magnesium-deficient

Table III

Calcium, Magnesium, and Phosphorus Content of Serum from Young Rats Which Had Been Restricted to the Low Magnesium Ration for Various Periods of Time

Initial age	Final age	Days on experimental diet	No. of animals	Mg. per 100 cc. serum			Ca:Mg
				Calcium	Magnesium	Phosphorus	
days	*days*						
25	25	0	9*	11.4	2.96	11.2	3.8
25	25	0	8	10.9	1.9	9.8	5.7
25	30	5	4*†	11.4	0.81	11.4	14.1
25	30	5	4†	11.5	0.71	9.3	16.2
25	35	10	5*	11.9	0.86	11.4	13.8
25	35	10	4	13.3	0.66	9.2	20.1
25	40	15	4*	12.7	0.86	8.2	14.9
25	40	15	2*‡	12.0	1.03	9.4	11.6
25	40	15	4	12.8	0.44	8.5	29.0
25	45	20	4*	13.5	1.2	9.2	11.2
25	45	20	4	12.6	0.43	6.7	29.3
25	50	25	4*	13.1	0.98	10.4	13.2
25	50	25	4	10.9	0.45	7.6	24.2
25	55	30	5	11.4	0.65	6.7	17.5
25	55	30	4*‡	12.2	1.1	7.6	11.1
25	56	31	5*	12.2	1.07	7.6	11.4
25	58	33	4*	12.2	0.90	7.6	13.5
25	58	33	3*‡	10.5	1.07	8.1	10.0

* Analyses of bones of these animals are presented in Table V.

† Animal sacrificed at height of vasodilatation.

‡ Animals sacrificed in first stage of induced convulsions.

ration led to an immediate and considerable decrease in the magnesium content of the serum, amounting on an average to 70 per cent within 5 days. By the 15th day the magnesium concentration of the serum had reached its minimum of 0.44 mg.; thereafter it continued to show subnormal values, practically all being less than 1 mg. per 100 cc. of serum. During convulsive seizures, which inevitably set in late in the survival period, the magnesium concentration underwent a sudden and sharp increase as contrasted with the persistence of low values in experimental mates free at that time from a convulsive condition; even during the attacks, however, the serum magnesium, in rising to such a value as 1.1 mg. per 100 cc. of serum, did not attain the normal level. The behavior of serum calcium in the rat under conditions of magnesium deficiency was consistent with what we previously recorded for the dog. Never falling below 10.9 mg. in the entire series, the values for serum calcium maintained their normal position throughout the survival period. Likewise the concentration of serum phosphorus showed no significant variation.

Equally valuable as the normal figure on blood chemistry, for similar reasons, are the data on the bone analyses of the control rats (Table IV). Indeed, the chemical changes in the bones of these normal young animals during growth deserve a detailed account. The dried bone added to its absolute weight with age, although the rate of increase was very irregular. Like the dried bone, the absolute amount of ash increased with age but to such a greater extent that the percentage ash showed mounting values. Even more striking was the progressive and rapid increase in the absolute amounts of calcium and magnesium in the bone, so that in 30 days both elements had quintupled in magnitude. Since they had increased at a more rapid rate than had the bone itself, and even the ash, the percentage of calcium and magnesium in the dried bone, as well as in the ash, showed a notable elevation over the same period of time. Although the absolute amount of phosphorus accumulated new increments with age, it did so to a lesser extent than either the bone or its ash, with effects manifested in gradual reduction of the percentage phosphorus in both materials. In brief, while the bone, ash, calcium, magnesium, and phosphorus were increasing in amount, the increase between them was so dissimilar that both in the bone and in the ash the percentage of cal-

TABLE IV

Absolute Weight of Bone and Its Ash, Together with Percentage Composition of Both with Respect to Calcium, Magnesium, and Phosphorus, in Young Rats Which Had Received the Control Ration for Various Periods of Time

Initial age	Final age	Days on control diet	No. of animals	Sex distribution	Mean absolute values per animal for femora, tibiæ, and fibulæ							Percentage of dried bones (femora, tibiæ, and fibulæ)				Percentage of ash		
					Bone	Ash	Ca	Mg	P	Ca:P	Ca:Mg	Ash	Ca	Mg	P	Ca	Mg	P
days	*days*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*									
25	25	0	9		180.5	79.4	17.7	0.5	17.4	1.01	35.0	44.0	9.8	0.28	9.6	22.4	0.64	22.0
25	30	5	5	♂ 3, ♀ 2	344.9	114.1	35.8	0.9	23.7	1.51	39.3	33.0	10.3	0.26	6.8	31.4	0.80	20.8
25	35	10	4	♂ 4	256.8	113.2	36.1	0.8	21.8	1.65	42.5	43.8	13.9	0.33	8.4	31.9	0.75	19.3
25	40	15	4	♂ 2, ♀ 2	392.6	184.2	52.4	1.5	40.1	1.53	38.0	46.9	14.9	0.40	10.2	31.9	0.84	20.8
25	45	20	4	♂ 2, ♀ 2	334.5	158.8	52.4	1.8	28.9	1.81	29.2	47.4	14.9	0.54	8.1	33.0	1.13	18.2
25	50	25	4	♂ 2, ♀ 2	362.0	173.9	52.5	1.8	30.0	1.74	29.0	47.9	14.5	0.50	8.3	30.3	1.04	17.3
25	55	30	4	♂ 2, ♀ 2	513.9	254.8	87.4	2.4	44.6	1.96	35.0	49.6	17.0	0.50	8.6	34.3	0.98	17.5

cium and magnesium increased while the percentage of phosphorus decreased with age.

Quite a different situation was presented in the bones of the animals restricted to the low magnesium diet, particularly as respects the changes in the absolute amounts of bone and its constituents. The bones showed steady gains in weight with age, which in this instance were so unusually rapid that almost from the beginning the bones of the animals on the deficient diet were heavier than those of the controls (Table V). Indeed as early as the 5th day on the deficient ration and thereafter for the duration of the survival period, the bones of these experimental animals contained more ash than did those of the controls. Despite the fact that these animals received an insignificantly small amount of magnesium, their bones showed throughout an increase in the absolute amounts of magnesium; but the increase was very slight, since retardation in the usual rate was evident by the 5th day on the diet and by the 30th day the absolute amount of magnesium was less than one-half of that in the controls. In view of the meager amount of magnesium in the bones of the animals subjected to the low magnesium diet, it is evident that no extraordinary deposition on its part can be responsible for the unusual increase in weight of the ash and in turn of the bone. Rather, the greater deposition of calcium brings about in large measure the increased weight of bone; for in these bones occurred 100.9 mg. of calcium as contrasted with 87 mg. in those of the controls. It is true that by increasing considerably in absolute amounts in the experimental animals phosphorus contributed to the greater density of the bone, but by showing a lesser increase than calcium it performs to that extent a subordinate part. As the result of magnesium deficiency, therefore, the bones and ash gained inordinately in weight, as judged by absolute increments; even more striking was the excessive deposition of calcium. It is most probable that the bones and ash became weightier through this accumulation of calcium in prodigious amounts.

Because the relative amounts of components, expressed in terms of percentage, constitute an extremely convenient method of visualizing the composition of bone, they convey most readily a definite impression of bone as it is seen in magnesium deficiency. In this condition the changed nature of the bone which is revealed

TABLE V

Absolute Weight of Bone and Its Ash, Together with Percentage Composition of Both with Respect to Calcium, Magnesium, and Phosphorus, in Young Rats Which Had Been Restricted to the Low Magnesium Ration for Various Periods of Time

Initial age	Final age	Days on experimental diet	No. of animals	Sex distribution	Mean absolute values per animal for femora, tibiæ, and fibulæ							Percentage of dried bones (femora, tibiæ, and fibulæ)				Percentage of ash		
					Bone	Ash	Ca	Mg	P	Ca:P	Ca:Mg	Ash	Ca	Mg	P	Ca	Mg	P
days	*days*				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*									
25	25	0	9		180.5	79.4	17.7	0.51	17.4	1.01	35.0	44.0	9.8	0.28	9.6	22.4	0.64	22.0
25	30	5	4	♂ 1, ♀ 3	285.4	145.8	51.1	0.64	27.4	1.87	80.0	51.0	17.9	0.22	9.6	35.1	0.44	18.8
25	35	10	5	♂ 3, ♀ 2	308.1	156.4	50.2	0.66	32.5	1.54	71.3	50.7	16.3	0.22	10.5	32.1	0.45	20.8
25	40	15	4	♂ 2, ♀ 2	381.9	195.9	66.2	0.65	40.7	1.62	102.4	51.3	17.3	0.16	10.6	33.8	0.33	20.8
25	40	15	2*	♂ 1, ♀ 1	355.4	191.2	65.5	0.31	36.5	1.82	214.3	53.8	18.4	0.086	10.2	34.3	0.16	18.8
25	45	20	4	♂ 2, ♀ 2	375.1	199.2	67.7	0.78	33.3	1.99	87.2	53.1	18.0	0.21	8.8	34.0	0.39	17.1
25	50	25	4	♂ 1, ♀ 3	500.5	276.3	98.4	0.86	47.2	2.08	115.0	55.2	19.6	0.17	9.4	35.6	0.31	17.1
25	55	30	4*	♂ 2, ♀ 2	591.0	313.5	107.8	0.75	54.2	1.99	143.3	57.2	18.2	0.13	9.1	34.4	0.24	17.3
25	56	31	5	♂ 2, ♀ 3	548.2	296.5	100.9	1.04	54.0	1.86	97.0	57.2	18.0	0.19	9.8	34.9	0.36	18.7
25	58	33	4	♀ 3, ♀ 1	573.5	312.1	103.3	1.40	54.6	1.89	73.5	52.6	18.0	0.18	9.5	33.1	0.45	17.5
25	58	33	3*	♂ 2, ♀ 1	556.9	289.2	110.4	0.80	55.7	1.98	138.0	53.2	19.8	0.14	10.1	37.2	0.27	18.8

* Animals sacrificed in first stage of induced convulsions.

by consideration of percentage composition becomes conspicuous through its lessened magnesium and greatly increased calcium content. While magnesium accumulated in these bones in a lesser amount than usual, the bone itself gained in weight beyond the usual amount; hence the per cent of magnesium in the bone dropped rapidly until it was, after 30 days on the diet, approximately one-half of its original figure and one-third of the figure normal for its particular age. On the other hand, the percentage of calcium in the bone at all ages was much increased over that of the controls. That this shift to a higher content of calcium in the bone is due, in the main, to actual absolute increase in this element and not to decreases in the other elements is manifest from the absolute figures which have already been presented. The percentage of phosphorus in the bone is likewise increased at all ages, but the increase is not so striking as that for calcium. For magnesium and calcium, substantially the same relationship prevails in the percentage composition of the ash, where they show respectively the decrease and increase; for phosphorus, however, the insignificant nature of its change makes its percentage not far removed from the normal figure. The net result of this diversity between the rate of bone growth and the deposition of its mineral elements is to give a bone that differs from normal not only in being heavier but in having a different composition; it contains a lessened percentage of magnesium, and a much greater percentage of calcium.

The end-effects of magnesium deficiency on the composition of bone are evident; the intermediate events have thus far not been touched. If the entire survival period of approximately 30 days be divided into three consecutive periods of 10 days each, it is seen that the increments for bone, ash, calcium, magnesium, and phosphorus, regarded individually without reference to each other, are not equal from period to period. This fact becomes more apparent when the figures are plotted on a semi-log grid which indicates the percentage rate of magnitude for the different periods (Chart I). This procedure is comparable to Hammett's determination of growth capacity (11, 12). From the slope of the curves, it is seen that bone weight and deposition of ash, as well as its constituents, calcium and phosphorus, were accelerated during the 1st days at a rate much in excess of that for the control bone. As a matter of fact, if we plotted for shorter intervals we would see that the acceleration is limited to the first 5 days on the diet; *i.e.*, between the ages 25 to 30 days. Thereafter the growth rates are closely parallel to, or very slightly less than those of the control bones, but the fact that the former bones were accelerated to such an extent during the first 5 days gives them a magnitude that is maintained for the remaining 25 days, even though the rate during this latter period has dropped to normal.

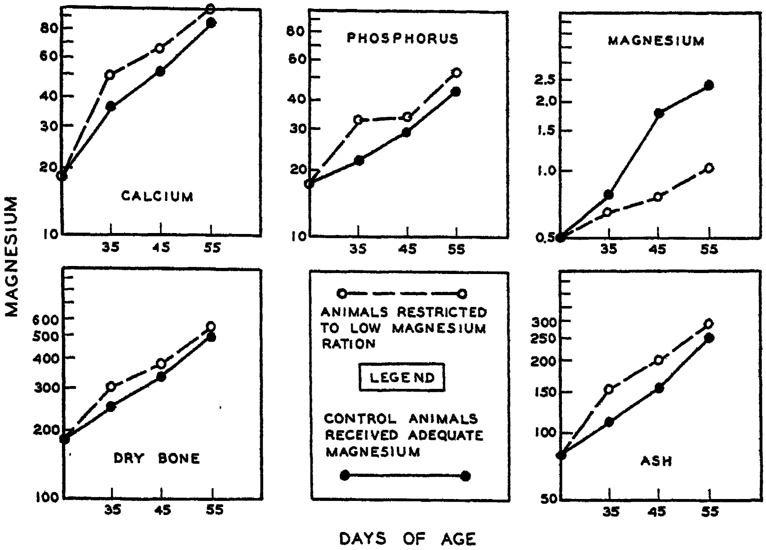


CHART I. The rates of increase in weight of bone and its constituents from young rats which had received the low magnesium ration as contrasted with the rates for young rats which had received the control ration.

In contrast to the accelerated growth of bone and extraordinary accumulation of ash, calcium, and phosphorus during the first 5 days on the diet, the rate of deposition of magnesium in the bone of the deficient animals was not only much slower but also much steadier. Its curve forms almost a straight line from the 25th to the 55th day of age, *i.e.* throughout the experimental period, but its slope is much lower than that for any other constituent. It should be mentioned, perhaps, that in the control animals magnesium differed from calcium and phosphorus in being deposited most rapidly when they were being deposited most slowly. Both in form and in slope the curve for magnesium deposition in the experimental animals is entirely different from that in the controls; when the intake of magnesium is markedly restricted, its accumulation in the bone is much more regular but very much slower.

When the composition of the bone is correlated with the chemical changes of the inorganic constituents of the blood in magnesium deficiency, it is seen that the magnesium concentration of the blood dropped at once, while the absolute amount of magnesium in the bone was slowly increasing. Nevertheless, the bone suffered from the condition in the blood, since the increase in magnesium in the former was so much retarded that the percentage magnesium in it and its ash was much reduced. Even more interesting was the behavior of magnesium in the blood and in the bone when the animal was sacrificed in the first stage of induced convulsions, that is within 2 minutes after the onset of the attack. During this limited time, magnesium rose in the blood and dropped sharply in the bone. Indeed the bones of these animals in every instance showed such a marked absolute decrease and lowered percentage of magnesium that the values were the lowest obtained.

DISCUSSION

That magnesium of the bone in normal animals increases progressively in absolute amount and in percentage during the early part of life is in keeping with the data from recent determinations. It is true that Wildt, following analyses on rabbits at birth and at 4 years of age, reached the conclusion that magnesium in bone diminished with age, but his work by the very nature of its plan would not reveal any detailed changes of the intervening period (13). From an investigation of the fresh bone of rats at intervals between 23 to 150 days of age, Hammett asserted that magnesium increased in absolute as well as in relative amount during this time (11), but the percentage of magnesium in the ash eventually declined after the animals reached 50 days of age (12). Although our immediate interest lies in the magnesium of the bone, it may not be amiss to cite the behavior of magnesium under normal conditions in the entire body. The absolute amount of magnesium in the body of the rat is said to be augmented from birth up to 90 days of age, and thereafter to be constant (14). The percentage of magnesium in the body at various ages is a matter in which there is a difference of opinion; on the one hand it is maintained that the percentage remains unchanged up to 40 weeks of age (15), on the other hand it is stated that, although the percentage is approximately constant up to 57 days of age (16), thereafter it diminishes (14). Even from such variable accounts, it is quite apparent that the course of deposition of magnesium in the bone does not necessarily parallel the course of retention of magnesium in the body. With allowance for difference in the choice of technique, the data, in the present paper, in so far as they extend, correspond in the main with those of Hammett in showing an increase in magnesium, both absolutely and relatively, in the bones of normal rats up to 50 days of age.

Under the stringent limitations imposed by a magnesium-deficient ration, the behavior of magnesium in bone is different. But even in this circumstance the magnesium of the bone rose from 0.51 mg. to 1.4 mg. in 33 days, an increase indicative of a tendency to lay down this element in so far as it was possible. The limiting factor was, of course, the almost complete lack of magnesium in the ration. Whether the viosterol in the diet exerted any additional effect in preventing the deposition of magnesium in the bone, or even in withdrawing it from this tissue, is problematic, although it must not be forgotten that McHargue and Roy reported that irradiation materially lessened the amount of magnesium in the bones and carcasses of rats (17). It is indeed extraordinary that, during 33 days on the magnesium-deficient ration, 0.89 mg. of magnesium was laid down in the bone, when the animal could not possibly have obtained from food during this time more than 0.3 mg. The most likely explanation of this deposition of magnesium in bone in the face of an almost negligible intake is a migration of magnesium to bone from such organs as liver, kidney, and heart where it is plentiful (18, 19). This accretion of magnesium in the bones of animals deprived of this element, an outcome which is indicative of a redistribution, suggests that bone has a stronger attractive force than any other tissue for magnesium.

Whether in normal or magnesium-deficient animals, the bone is not dominant in the distribution of magnesium throughout the body. During strong muscular contractions in normal animals, magnesium is released from muscle into the blood stream; for a lowered content of the element in muscle accompanies the heightened content in the serum (20). To what extent the transfer between muscle and serum occurs in moments of even more intense activity in magnesium deficiency is unknown; the transfer between bone and serum at these times of particular stress in this disorder is, however, more than likely. From the lowered serum magnesium, which appeared early and persisted all the while until the onset of convulsions, it is evident that the bone had not yielded its entire stock of magnesium to maintain a normal level in the blood. Instead the withdrawal or diversion of magnesium from the bone, in keeping the concentration of this element at even the low level in the serum, was slow and steady. In a crisis, however, magnesium was immediately mobilized from the bone, and perhaps from other tissues. This event is indicated in our data when, in the first stage of induced convulsions, the raising of blood magnesium occurred simultaneously with the lowering of bone magnesium. When it is remembered that the onset of convulsions is measured in seconds, the speed of transport of magnesium from the bone to the blood is truly rapid. Moreover, it is noteworthy that, in this rapid mobilization, the withdrawal of magnesium is independent of any disturbance in bone calcium. If magnesium is so intimately associated with calcium in bone structure, as it has been generally regarded, the rapid removal of the one without disturbance of the other is a phenomenon requiring elucidation. We are neither inclined nor prepared to take exception to the preexisting notions on the structure of bone, but we are desirous of calling attention, without undue emphasis or subscription, to an extreme theory on this subject. In his conception of the composition of bone as consisting principally of half-basic calcium phosphate with a slight admixture of calcium carbonate, Klement takes no account of magnesium as a true component. Magnesium, he says, is present in the fluid of the micelles; therefore it is always obtained in the ash of bone, it has always been determined as an integral part of bone, and indeed on the basis of analyses it could not be distinguished from the actual constituents of bone. When on other grounds he thus came to view magnesium as an adventitious element, he inferred forthwith that it was not an inherent part of the structure of bone (21).

With respect to the effect of a restricted magnesium regimen on calcium metabolism, we considered in the preceding paper the much debated proposition that magnesium and calcium exhibit a mutually antagonistic action. Although the interaction is said to be manifested by characteristic changes in the neuromuscular response, in inorganic metabolism, and in bone composition, we canvassed the data in the literature from the standpoint of the metabolic effects alone, in which the administration of the one element is purported to lead to enhanced elimination of the other, and from this limited aspect of the subject we gained the impression that the evidence on the mutuality of the action was inconclusive. Nevertheless, we did record the majority view that there was a physiological relationship and that it was unidirectional; magnesium exercises an influence over calcium. In bone the mutual character of the antagonistic action again presents itself; for here, according to the numerous citations in the literature, magnesium and calcium display an inverse relationship. If this inference is true, it suggests four possible ways of modifying bone with the resulting composition being one of two kinds. Theory demands that, on the one hand, a high intake of calcium should lead to a bone rich in calcium but poor in magnesium; a restricted intake of magnesium should produce the same effect. On the other hand, a ration high in magnesium should lead to a bone rich in magnesium but poor in calcium; a ration low in calcium should produce the same effect. Before presenting the array of evidence on all four possibilities, we may admit at once that there are gaps and contradictions. Particularly the effects of ingesting large amounts of either calcium or magnesium salts abound in controversy, to some extent because the protracted experimental period necessary for the production of altered bone gives time for gastrointestinal disturbances, but largely because chemical factors, such as phosphorus, enter into any interplay between the calcium and magnesium cycles.

When the reported effects of varying amounts of calcium intake on the composition of bone, and especially its magnesium content, are compiled, it is seen that rations excessive in calcium introduce at least one of the aforementioned complications. If the theory of antagonism held without exception, it would be expected that a ration containing an exorbitant amount of calcium would induce a high calcium and a low magnesium content in the bone. But in the light of investigations on rickets, it is now known that increasing the moiety of calcium in the diet, unless the level of phosphorus is commensurately raised, actually gives rise to a bone poor in calcium, due to a disproportion between calcium and phosphorus. So far as we know, the effect which a ration high in calcium, together with proportionately increased phosphorus, produces on the magnesium content of bone has not been described. One investigation in a comparable category deals with the effect on the calcium and magnesium content of the entire body; rats receiving an excess of calcium incorporated an increased percentage of calcium and a decreased percentage of magnesium in their tissues (16). In the case of rations deficient in calcium, on the contrary, the effects on bone composition are decisive in nature and singularly free from controversial points. When animals incur a calcium deficit by restriction to a low calcium regimen (22, 23), or sustain an irregularity in their calcium metabolism from parathyroidectomy (24, 25), both conditions conducive to lowered serum calcium, their bones invariably contain a lessened amount of calcium and an increased amount of magnesium. Furthermore, in humans suffering from rickets or osteomalacia, the bones are characterized by a similarly lowered calcium and increased magnesium content (26, 27). The argument for the antagonistic action of calcium towards magnesium is more impressive from these data on bone than it was from the metabolic reports, probably because the experiments relating to bone were of necessity prolonged to afford opportunity for a distinct change, whereas the metabolic studies were often terminated after a single injection of a calcium salt and observations limited to a few hours.

On the converse side of this relationship between the two elements, the principle that magnesium exerts a physiological control over calcium seemed quite conclusively established from metabolic evidence. Unfortunately, according to published accounts, the effect of immoderate ingestion of magnesium salts on bone composition is attended by even more complexity than that of excessive calcium, since toxicity and anorexia are said to be disturbing factors which obscure the true state (28, 29). Yet, in not a few investigations, the administration of diets excessive in magnesium salts resulted in a lessened amount of calcium in the bones (30–33) and in the body (34). From the conclusion that magnesium manifests its antagonistic action, among other ways, by lowering the calcium content of bone, the belief was thus derived that magnesium exercises a specific inhibitory effect on calcification. The rationale of the mechanism was sought on a physicochemical basis, and thereby was revealed another intricacy in the calcium to magnesium relation. By means of model experiments it was demonstrated that magnesium operates against calcification (35–37) largely by increasing the solubility of calcium salts (35, 38); but that this anticalcification, or it may be decalcification, disappears with an increased phosphorus concentration of the solution (36–38). This protective agency of phosphorus has received confirmation *in vivo* (32). In proper amount, phosphorus apparently offsets the effects of excessive magnesium as completely as those of excessive calcium intake; accordingly it is a factor to be recognized in the counter relationship between calcium and magnesium. Of the two complicating influences which may operate in the overingestion of magnesium, namely gastrointestinal disturbance and a high phosphorus level, the former depreciates in several instances the validity of the results *in vivo* whereby magnesium is seemingly antagonistic to calcium; for it is difficult to ascertain how much of the lowered content of calcium in the bone is due to the inhibitory action of magnesium and how much to anorexia. The evidence *in vitro* is free, perchance, from such an entanglement, and is therefore the more convincing. Here the effect of magnesium in checking calcification is not only demonstrated but also explained. All in all, the bone data consequent to high magnesium intake, although less conclusive perhaps than the evidence from metabolic experiments, square with the proposition that magnesium does exert control over calcium.

In accordance with the fourth possible arrangement of calcium and magnesium intake capable of revealing their antagonism through an alteration in bone, the present investigation has shown that a ration deficient in magnesium brings about an increase in the weight of bone and its calcium content. It may be mentioned that a low magnesium regimen apparently affects the calcium content of the entire body in the same manner as it does that of the bone. As the result of restricting rats for 20 days to a magnesium-deficient diet, Medes determined that the percentage of calcium in the body was definitely increased above normal while the percentage of magnesium was decreased (16). With this complete agreement between the effects of low magnesium on the calcium content of the body and of the bone, the evidence points unmistakably to a relationship between magnesium and calcium which in its character fulfils the provisions of theory. Just as excess magnesium inhibits calcification, inadequate magnesium gives rise to increased calcification. In view of the marked retention of calcium resulting from magnesium deficiency (5), it now becomes apparent that the bone is the site of deposition of much of the retained calcium. Thus the metabolic and bone studies with a low magnesium regimen are at one in upholding the view that magnesium acts upon calcium.

In the foregoing survey we considered the relevant data on bone, dealing with the effects of calcium and magnesium upon each other. We shall not, however, attempt to draw any conclusions on the mutuality of the relationship between the two elements and on the control of calcium over magnesium. These topics have served their purpose in contributing plausibility, so much as they have, to the general proposition that calcium and magnesium are physiologically related. Our immediate interest is whether magnesium can govern calcium, and all the evidence tends to give an affirmative answer to this question. The physicochemical considerations whereby magnesium is said to be able to displace calcium from its union with protein or amino acid, and to increase the solubility of the calcium salts; the metabolic experiments in which low magnesium led to calcium retention, and high magnesium to calcium excretion; and now the bone studies in which an excess of magnesium induced decalcification, while a deficiency of magnesium induced hypercalcification—all these lines of evidence are conducive to the view that magnesium holds physiological sway over calcium.

The fact that the remarkable acceleration in calcification is accomplished in the first 5 days on the diet and is then checked, just as the excessive retention of calcium by the dogs on the diet is stopped at a comparable time, is open to at least two explanations. The rats were 30 days of age when the extraordinary acceleration in calcification ceased. When Hammett observed that the "growth capacity" of calcium in normal animals was disturbed at this age, he attributed it to the onset of puberty (10, 11). Perhaps that interpretation holds here. On the other hand, the cessation of the accelerated calcification in the animals on low magnesium diets at this particular time might be due to the onset of nutritive failure, similar to that which occasioned a reversal of calcium retention in the dogs. In this case, the animals succumb to convulsions before the bone is severely depleted of calcium.

SUMMARY

In animals restricted to a low magnesium regimen, the bone was unusually heavy and overly abundant in its absolute content of ash, calcium, and phosphorus; the increased weight of the bone was due in large measure to the excessive deposition of calcium. While the bone itself gained in weight at an extraordinary rate, the accumulation of calcium so exceeded this as to give a bone that differed from normal not only in being heavier but also in having a different composition; *viz.*, a greater percentage of calcium.

From a study of the rate of increase in magnitude during different arbitrary periods, it is seen that the acceleration for bone and calcium was limited to the first 5 days on the diet; thereafter the rates were almost parallel to those of the control bones. Thus the heaviness of bone and its intensive calcification, which was imparted by the initial acceleration, was maintained throughout the survival period. These changes are attributed, with certain limiting circumstances, to the physiological sway of magnesium over calcium. The cessation of this influence, after its early and comparatively brief period of operation, is open to either or both of two interpretations: the onset of puberty or nutritive failure.

Although the animals received an insignificantly small amount of magnesium, their bones showed an accumulation of this element which was much less than usual but which represented a definite absolute gain over the entire period; accordingly this accretion of magnesium in bone under conditions of deprivation indicates a redistribution of the element within the body. The rate of deposition of magnesium in the bone throughout the entire period of the deficiency, while steadier, was much slower than that of the other components, so that it contributes to the altered composition of the bone with its lowered percentage of magnesium. During the few seconds that passed before the first stage of induced convulsions was reached, the magnesium level rose in the blood and dropped sharply in bone. This rapid mobilization of magnesium takes on interest in connection with the composition of bone.

BIBLIOGRAPHY

1. Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, **96**, 519 (1932).
2. Orent, E. R., Kruse, H. D., and McCollum, E. V., *Am. J. Physiol.*, **101**, 454 (1932).
3. Kruse, H. D., Schmidt, M. M., and McCollum, E. V., *Am. J. Physiol.*, **105**, 635 (1933).
4. Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, **100**, 603 (1933).
5. Kruse, H. D., Schmidt, M. M., and McCollum, E. V., *J. Biol. Chem.*, **106**, 553 (1934).
6. Erdtman, H., *Z. physiol. Chem.*, **177**, 211, 231 (1928).
7. Fell, H. B., and Robison, R., *Biochem., J.*, **24**, 1905 (1930).
8. Wells, H. G., *Arch. Int. Med.*, **7**, 721 (1911).
9. Bethke, R. M., Steenbock, H., and Nelson, M. T., *J. Biol. Chem.*, **58**, 71 (1923–24).
10. Hammett, F. S., *J. Biol. Chem.*, **64**, 409 (1925).
11. Hammett, F. S., *J. Biol. Chem.*, **64**, 685 (1925).
12. Hammett, F. S., *J. Biol. Chem.*, **64**, 693 (1925).
13. Wildt, E., *Landw. Versuch.-Stat.*, **15**, 404 (1872); *Jahresber. Fortschr. Tierchem.*, **2**, 266 (1874).
14. Medes, G., and Humphrey, G. J., *J. Biol. Chem.*, **74**, 149 (1927).
15. Buckner, G. D., and Peter, A. M., *J. Biol. Chem.*, **54**, 5 (1922).
16. Medes, G., *J. Biol. Chem.*, **68**, 295 (1926).
17. McHargue, J. S., and Roy, W. R., *Am. J. Physiol.*, **92**, 651 (1930).
18. Aloy, J., *Compt. rend. Soc. biol.*, **54**, 604 (1902).
19. Javillier, M. M., *Bull. Soc. chim. biol.*, **12**, 709 (1930).
20. Wacker, L., *Klin. Woch.*, **8**, 244 (1929).
21. Klement, R., *Z. physiol. Chem.*, **196**, 140 (1931).
22. Toverud, G., *J. Biol. Chem.*, **58**, 583 (1923–24).
23. Weiser, S., *Biochem. Z.*, **66**, 95 (1914).
24. Hammett, F. S., *J. Biol. Chem.*, **57**, 285 (1923).
25. Hammett, F. S., *J. Biol. Chem.*, **72**, 527 (1927).
26. Gassmann, T., *Z. physiol. Chem.*, **70**, 161 (1910).
27. McCrudden, F. H., *J. Biol. Chem.*, **7**, 199 (1909–10).
28. Elmslie, W. P., and Steenbock, H., *J. Biol. Chem.*, **82**, 611 (1929).
29. Weiske, H., *Z. Biol.*, **31**, 421 (1895).
30. McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., unpublished data, cited by Park, E. A., *Physiol. Rev.*, **3**, 129 (1923).

31. Mouriquand, G., Leulier, A., and Roche, A., *Compt. rend. Soc. biol.*, **107**, 676 (1931).
32. Villuis, F., *Compt. rend. Soc. biol.*, **110**, 469 (1932).
33. Meyer zu Horste, G., *Klin. Woch.*, **11**, 1796 (1932); *Monatschr. Kinderh.*, **54**, 203 (1932).
34. Malcolm, J., *J. Physiol.*, **32**, 183 (1905).
35. Holt, L. E., Jr., La Mer, V. K., and Chown, H. B., *J. Biol. Chem.*, **64**, 509 (1925).
36. Kramer, B., Shelling, D. H., and Orent, E. R., *Bull. Johns Hopkins Hosp.*, **41**, 426 (1927).
37. Shelling, D. H., Kramer, B., and Orent, E. R., *J. Biol. Chem.*, **77**, 157 (1928).
38. Forbes, J. C., *J. Biol. Chem.*, **93**, 255 (1931).

SYNTHESIS OF THE PHOSPHORIC ESTERS OF HYDROXYAMINO ACIDS

III. RESOLUTION OF *d,l*-SERINEPHOSPHORIC ACID AND SYNTHESIS OF *l*-HYDROXYPROLINEPHOSPHORIC ACID

By P. A. LEVENE and A. SCHORMÜLLER

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, June 29, 1934)

In previous communications of this series[1, 2] the synthesis of the phosphoric esters of two hydroxyamino acids (namely natural tyrosine and *d,l*-serine) was reported. It was desired to prepare the phosphoric esters of the other naturally occurring hydroxyamino acids.

The present communication contains a report on the synthesis of the phosphoric ester of the natural *l*- (levo-)hydroxyproline. This substance was prepared by the method which led to the formation of the ester of *d,l*-serine.

At the same time, we wish to state that the method was not applicable to the synthesis of the phosphoric esters of either hydroxyaspartic or hydroxyglutamic acids. In fact, up to the present, we have failed to prepare either of these esters by any other method of phosphorylation which we have applied.

The present paper also contains a report on the resolution of *d,l*-serinephosphoric acid to give dextro-serinephosphoric acid, the maximum rotation of which was found practically identical with that of the naturally occurring substance. The purity of the substance described by Lipmann and Levene[3] is thus definitely established.

Occasion was also taken to convert the dextro- (*d*-)phosphoser-

---

[1] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **100**, 583 (1933).

[2] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, **105**, 547 (1934).

[3] Lipmann, F. A., and Levene, P. A., *J. Biol. Chem.*, **98**, 109 (1932).

ine into levo-phosphoglyceric acid. The ease with which this reaction is accomplished indicates that the same reaction may occur in the living organism and, in view of the part played by phosphoglyceric acid in carbohydrate metabolism, a new physiological significance may be attributed to phosphoproteins.

Incidentally, mention may be made of the use of Raney's catalyst for the synthesis of aminomalonic ester and hydroxyglutamic acid. In the latter case the method of Harington[4] and Randall[4] is more advantageous from the view-point of yield; on the other hand, by Raney's method, the isolation of the intermediate product—the dihydropyrazine derivative—was accomplished without difficulty.

EXPERIMENTAL[5]

Dextro-Serinephosphoric Acid—The *d,l*-serine required for the preparation of *d,l*-serinephosphoric acid was prepared essentially according to the method of Leuchs and Geiger.[6] However, instead of the chloroacetal, the bromoacetal (obtained from paraldehyde with bromine) was treated with sodium ethylate on the steam bath,[7] thereby producing the ethoxyacetal by a much simpler process and with a very good yield. The *d,l*-serine (200 gm.) was phosphorylated[2] in portions of 8 gm. and yielded 106 gm. of the barium salt of *d,l*-serinephosphoric acid.

Two portions of the barium salt (50 gm. each) were each suspended in 400 cc. of water. Sulfuric acid was added in slight excess and the mixture vigorously shaken mechanically during 1 hour. The solutions were combined, filtered, and quantitatively freed from barium and sulfate ions. The clear solution was made faintly alkaline by addition of a concentrated solution of brucine in methyl alcohol. The excess brucine was then removed by extraction with chloroform and the aqueous layer evaporated under diminished pressure to a thick syrup.

The product was dissolved in 750 cc. of boiling methyl alcohol and the solution cooled in ice water during 2 hours. On filtering through a Buchner funnel, there were obtained 175 gm. of nicely crystallized brucine salt. On concentrating and standing in the refrigerator the mother liquor yielded an additional 40 to 50 gm. of the salt.

---

[4] Harington, C. R., and Randall, S. S., *Biochem. J.*, **25**, 1917 (1931).

[5] We wish to thank Dr. R. S. Tipson for his kindness in assisting in the preparation of the manuscript.

[6] Leuchs, H., and Geiger, W., *Ber. chem. Ges.*, **39**, 2644 (1906).

[7] Späth, E., *Monatsh. Chem.*, **36**, 4 (1915).

175 gm. of the first crop of brucine salt were recrystallized four times from absolute methyl alcohol and a small sample then converted into the barium salt. The optical rotation of the barium salt, dissolved in 10 per cent hydrochloric acid, was

$$[\alpha]_D^{25} = \frac{+0.75° \times 100}{2 \times 6} = +6.3°$$

After seven recrystallizations, the rotation under the same conditions was +8.6°. After nine recrystallizations, the rotation was constant (+9.4°) and was not changed by further crystallization. The yield was 40.5 gm.

Dried under diminished pressure at 60°, the dibrucine salt had the following composition.

5.940 mg. substance: 0.382 cc. N_2 (757 mm. at 26°)
4.190 " " : 8.980 mg. molybdate
$C_{49}H_{60}O_{14}N_5P$.* Calculated. N 7.20, P 3.19
973.5 Found. " 7.31, " 3.11

* In our former paper[8] the formula and calculated values for the dibrucine salt should be corrected.

On heating, the salt sinters at 100° and decomposes[8] around 130°.

The barium salt, obtained from the brucine salt with barium hydroxide, had the following composition after drying at 110°.

10.580 mg. substance: 0.387 cc. N_2 (762 mm. at 26°)
3.472 " " : 23.180 mg. molybdate
$C_3H_6O_6NPBa$. Calculated. N 4.36, P 9.65
320.4 Found. " 4.18, " 9.69

For the determination of the specific rotation, a 6 per cent solution of the barium salt in 10 per cent hydrochloric acid was used.

$$[\alpha]_D^{25} = \frac{+1.13° \times 100}{2 \times 6.0} = +9.4°$$

Calculated as the free acid, the rotation[3] was

$$[\alpha]_D^{25} = \frac{+1.13° \times 100}{2 \times 3.47} = +16.3°$$

---

[8] Levene, P. A., and Schormüller, A., *J. Biol. Chem.*, 103, 537 (1933).

Phosphate of l-Glyceric Acid from d-Serinephosphoric Acid—To a suspension of 4.8 gm. of finely powdered barium salt of *d*-serinephosphoric acid in 100 cc. of water 2.7 gm. of concentrated sulfuric acid were added and the mixture was vigorously shaken during 30 minutes. To this suspension a concentrated aqueous solution of 2.3 gm. of pure crystallized barium nitrite ($Ba(NO_2)_2 \cdot H_2O$) was slowly added during 1 hour, being cooled in ice throughout the operation. The mixture was then allowed to stand overnight at room temperature.

A small portion of the liquid was now concentrated under diminished pressure, rendered faintly alkaline by addition of barium hydroxide, and filtered through charcoal. On rapidly heating the solution to boiling, the barium salt was precipitated and was filtered off while hot. After reprecipitating with alcohol from an aqueous solution made faintly acid to Congo red[9] the substance still contained 1.5 per cent of nitrogen.

Therefore, the larger part of the reaction liquid was treated once more with 1.2 gm. of sulfuric acid and 2.3 gm. of barium nitrite as described above. Finally, after standing for 3 to 4 hours, the barium salt was prepared. The yield was 1.4 gm.

The substance was dried at 110° for analysis and had the following composition.

5.005 mg. substance: 2.120 mg. CO_2 and 0.980 mg. H_2O
4.550 " " : 29.900 " molybdate
40.470 " " : 27.820 " $BaSO_4$

$C_3H_5O_7PBa$.	Calculated.	C 11.20, H 1.55, P 9.64, Ba 42.75
321.4	Found.	" 11.55, " 2.19, " 9.54, " 40.45

For the determination of the specific rotation, Vogt's[9] method was followed. 0.4018 gm. of the barium salt was dissolved in 2.5 cc. of N sulfuric acid and immediately neutralized with 2.5 cc. of N sodium hydroxide. The rotation was

$$[\alpha]_D^{25} = \frac{-1.00° \times 100}{2 \times 8.036} = -6.2°$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{-1.00° \times 100}{2 \times 4.65} = -10.7°$$

---

[9] Vogt, M., *Biochem. Z.*, **211**, 1 (1929). Neuberg, C., Weinmann, F., and Vogt, M., *Biochem. Z.*, **199**, 248 (1928).

After the addition of a few drops of concentrated hydrobromic acid the rotation changed,[9] and after standing for 3 days it was

$$[\alpha]_D^{25} = \frac{+0.39° \times 100}{2 \times 8.036} = +2.4°$$

Dextro-serine, converted into glyceric acid, yields the levo acid.[10]

Levo-Hydroxyprolinephosphoric Acid—Phosphorus pentoxide (4.0 gm.) was dissolved in 40 gm. of 100 per cent phosphoric acid. To this solution were added 5.2 gm. of pure hydroxyproline (very finely powdered) and the mixture was thoroughly stirred. After standing in a desiccator during 50 to 60 hours, finely chopped ice and then 2 liters of ice water were added. 180 to 200 gm. of crystallized barium hydroxide and a little phenolphthalein were now added and the mixture was vigorously stirred until it became pink in color. The barium phosphate was removed by centrifuging and the precipitate well stirred with about 1 liter of water and filtered through a thin layer of charcoal. The combined solutions were rendered neutral by addition of a few drops of hydrobromic acid and then concentrated under diminished pressure at 40° to a volume of 150 to 200 cc. The solution was made faintly alkaline with barium hydroxide, filtered through charcoal, and alcohol was added until the concentration of alcohol was about 60 per cent. The thick precipitate was readily separated by centrifuging. After washing successively with 50 per cent alcohol, 80 per cent alcohol, alcohol-ether 1:1, and anhydrous ether, the barium salt was filtered off and dried. Yield 4.3 gm.

The barium salt is a very fluffy, white powder which is easily soluble in cold water. On heating the aqueous solution, the barium salt separates as a compact, crystalline precipitate, which consists of imperfectly formed feather-like crystals.

For analysis, the salt was dissolved in a little cold water, precipitated by boiling the solution, redissolved in cold water, and finally precipitated with alcohol. After drying at 80° under diminished pressure, it still contains 1 mole of water of crystallization which can be driven off by drying at 130° under a high vacuum during 24 hours.

145.5 mg. substance lost 8.0 mg. H_2O

$C_5H_8O_6NPBa \cdot H_2O$.	Calculated.	H_2O 4.9
364.5	Found.	" 5.5

---

[10] Fischer, E., and Jacobs, W. A., *Ber. chem. Ges.*, 40, 1068 (1907).

The barium salt, dried at 80°, had the following composition.

7.190 mg. substance: 4.185 mg. CO_2 and 2.090 mg. H_2O
8.696 " " : 0.297 cc. N_2 (754 mm. at 27.5°)
4.004 " " : 23.450 mg. molybdate
0.2200 gm. " : 0.1405 gm. $BaSO_4$
$C_5H_8O_6NPBa \cdot H_2O$ (364.5)
Calculated. C 16.48, H 2.7, N 3.84, P 8.52, Ba 37.64
Found. " 16.06, " 3.2, " 3.85, " 8.51, " 37.58

For the determination of the specific rotation, the barium salt was dried at 130° and dissolved in 10 per cent hydrochloric acid.

$$[\alpha]_D^{25} = \frac{-1.60^\circ \times 100}{2 \times 6.0} = -13.3^\circ$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{-1.60^\circ \times 100}{2 \times 3.656} = -21.9^\circ$$

Brucine Salt of Hydroxyprolinephosphoric Acid—3.6 gm. of the barium salt of the hydroxyprolinephosphoric acid were dissolved in a little water and the barium quantitatively removed by the addition of about 10 cc. of 10 per cent sulfuric acid. To the aqueous solution a solution of brucine in methyl alcohol was added until the mixture showed a distinctly alkaline reaction to litmus. The excess of brucine was extracted with chloroform and the aqueous layer evaporated to dryness under diminished pressure at 40°.

The residue was dissolved in a little hot butyl alcohol (about 75 per cent) and allowed to stand in the refrigerator overnight. The brucine salt crystallized out in rosettes of large needles.

These crystals were dried over phosphoric anhydride at room temperature and then on further drying at 130° in a high vacuum during 30 hours, 5 moles of water were lost. The dry substance had an indefinite melting point between 180–183°. It had the following composition.

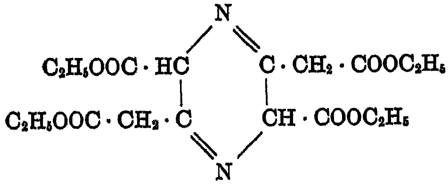
7.965 mg. substance: 0.483 cc. N_2 (756 mm. at 25°)
3.810 " " : 9.500 mg. molybdate
$C_{51}H_{62}O_{14}N_5P$. Calculated. N 7.01, P 3.11
999.5 Found. " 6.90, " 3.60

Diethyl Ester of Aminomalonic Acid[11]—25 gm. of isonitrosomalonic ester (purified by distillation under diminished pressure) were dissolved in 45 gm. of absolute alcohol, 5 gm. of Raney's nickel catalyst were added, and the mixture was shaken with hydrogen at a pressure of 40 pounds per sq. inch. After 4 to 5 hours, the absorption of hydrogen ceased. The catalyst was removed by centrifuging and the alcohol evaporated off under diminished pressure. The syrup was mixed with 250 cc. of anhydrous ether and saturated with dry hydrogen chloride. After standing for 2 hours in the refrigerator, the thick paste was filtered through a Buchner funnel and the product washed with dry ether. Yield 18 to 21 gm.

For analysis it was dissolved in a small amount of absolute ethyl alcohol containing 1 per cent of dry hydrogen chloride and precipitated by the addition of warm ether. The melting point was 170°.

10.610 mg. substance: 0.608 cc. N_2 (759 mm. at 24°)
5.746 " " : 3.800 mg. AgCl
$C_7H_{14}O_4NCl$. Calculated. N 6.62, Cl 16.75
211 Found. " 6.57, " 16.36

Isolation of Diethyl Ester of 2, 5-Dicarbethoxy-Dihydropyrazine-3,-6-Diacetic Acid (I) in Preparation of Hydroxyglutamic Acid[4]



I

25 gm. of ethyl acetone dicarboxylate in 75 cc. of absolute alcohol were shaken in the presence of Raney's nickel catalyst with hydrogen at a pressure of 40 pounds per sq. inch until the absorption had ceased (3 to 4 hours) and the solution was then centrifuged in order to remove the catalyst. After standing overnight in a well stoppered bottle at room temperature, the brown liquid was cooled

---

[11] Piloty, O., and Neresheimer, J., *Ber. chem. Ges.*, **39**, 514 (1906). Putochin, N. J., *Ber. chem. Ges.*, **56**, 2213 (1923).

in ice for several hours. During this time, a thick paste of golden-yellow needles formed.

The ester crystallized from absolute alcohol in long, bright yellow needles having a melting point of 124°. For analysis, it was twice recrystallized from absolute ethyl alcohol and dried at 60° under diminished pressure. It had the following composition.

4.465 mg. substance: 8.870 mg. CO_2 and 2.580 mg. H_2O
7.960 " " : 0.515 cc. N_2 (759 mm. at 25°)

$C_{18}H_{26}O_8N_2$.	Calculated.	C 54.24, H 6.6, N 7.04
398.2	Found.	" 54.17, " 6.5, " 7.40

Conversion of (I) into 3,6-Dimethylpyrazine—The method given by Harington and Randall[4] was followed exactly. The dimethylpyrazine was purified by isolation of the mercuric chloride compound. This was converted into the picrate, which crystallized from absolute alcohol in long lemon-yellow needles and had a melting point of 157°.

MONOACETONE *d*-XYLOKETOSE

A PRELIMINARY NOTE

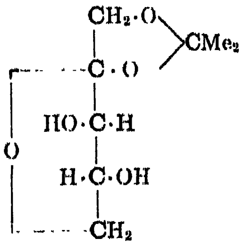
BY P. A. LEVENE AND R. STUART TIPSON

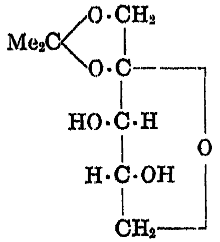
(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

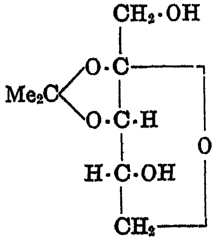
(Received for publication, July 2, 1934)

Whereas in the case of hexoses both the aldoses and the ketoses have been the subject of extensive investigation, in the case of pentoses the *keto* forms have attracted practically no attention, since they are difficultly accessible either as natural products or by methods of synthesis. Of the four possible forms only one has been discovered in nature—the *keto*xylose isolated from urine, in cases of pentosuria, by Levene and La Forge.[1] The *d* form of this sugar was recently synthesized by Schmidt and Treiber[2] and a detailed study of its properties and of its derivatives is now being undertaken in our laboratory. The present note deals with the preparation of the monoacetone derivative.

d-Xyloketose was condensed with acetone in the presence of sulfuric acid and anhydrous copper sulfate to give a crystalline monoacetone derivative which does not exhibit mutarotation in water. It therefore presumably has one of the three following structures.







---

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 18, 319 (1914).

[2] Schmidt, O. T., and Treiber, R., *Ber. chem. Ges.*, 66, 1765 (1933).

Investigation of its precise structure (and of that of the glycosides of *d*-xyloketose) is now in progress.

EXPERIMENTAL

Preparation of Monoacetone d-Xyloketose—*d*-Xyloketose was prepared from *d*-xylose as described by Schmidt and Treiber.[2] 2 gm. of pure xyloketose (regenerated from the *p*-bromophenylhydrazone) were shaken with a mixture of 0.04 cc. of concentrated sulfuric acid, 40 cc. of acetone (analytical reagent), and 4 gm. of anhydrous copper sulfate at room temperature (27°) during 90 hours.

The product was isolated as for monoacetone ribose,[3] giving a pale yellow syrup (yield, 2 gm.) which was purified by distillation under a high vacuum.

The substance boiled at 130–140° (bath temperature) at 0.1 mm. and was collected as a colorless, viscous syrup which quickly crystallized spontaneously to a solid mass.

It had the following composition.

4.673 mg. substance: 8.665 mg. CO_2 and 3.180 mg. H_2O

$C_8H_{14}O_5$.	Calculated.	C 50.50,	H 7.4
	Found.	" 50.56,	" 7.6

The crystalline material was somewhat hygroscopic and had a melting point of 50–52° (with softening at 45°) in a sealed tube.

Its specific rotation was

$$[\alpha]_D^{28} = \frac{-0.10° \times 100}{2 \times 1.930} = -2.6° \text{ (in water, 2 minutes after solution)}$$

The rotation remained unchanged after the addition of 1 drop of concentrated ammonia.

---

[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933).

A STUDY ON KERATIN

BY DAVID R. GODDARD* AND LEONOR MICHAELIS

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, May 16, 1934)

Keratins are the proteins of epidermal and skeletal tissues which are insoluble in the usual protein solvents, not digested by trypsin or pepsin, and high in cystine content. Such a definition intentionally excludes fibroin, the major protein of silk. It will be shown in this paper that keratins can be converted into proteins soluble in alkali or acid, with a definite optimum pH of flocculation (which may be interpreted as an isoelectric point), and digestible by trypsin or pepsin. This is accomplished by breaking the disulfide bonds of the protein. Papers on the oxidation of keratins have been published by Lissizin (1), Stary (2), and Waldschmidt-Leitz (3), who used bromine, permanganate, and H_2O_2 as oxidants. Stary and Waldschmidt-Leitz have shown that the oxidized keratin is digested by trypsin. The oxidizing agents are not specific for the disulfide groups, but attack the protein molecule at other points, and they act very slowly. In contrast, the reductants will be shown to act very quickly and without bringing about any other appreciable chemical alteration than that concerned with the sulfur. These agents dissolve keratin only at alkaline reaction (pH 10 to 13), but the action is not due to alkali alone. Products prepared from the solutions behave as true proteins, and not as products of hydrolysis. Their solutions are precipitated by ordinary protein precipitants such as sulfosalicylic acid and lose this property when digested by trypsin or pepsin.

Reductants available for reduction of disulfide groups are thioglycolic acid, potassium cyanide, sodium sulfide, and sodium sulfite. The chemical process exhibited by these reagents on simple disulfide compounds such as cystine can be formulated as follows:

---

* National Research Council Fellow in the Biological Sciences.

(1) $R—S—S—R + 2HS—CH_2COOH \rightarrow 2R—SH + [S—CH_2COOH]_2$ (see (4))*
(2) $R—S—S—R + HCN \rightarrow R—SH + R—S—CN$ (see (5))
(3) $R—S—S—R + H_2S \rightarrow R—SH + H_2S_2$†
(4) $R—S—S—R + H_2SO_3 \rightarrow R—SH + R—S—S—O_3H$ (see (6, 7))

Reactions 1 to 3 occur only in slightly alkaline solution; Reaction 4 is most rapid in neutral to mildly acid solutions.

When keratin, such as wool or feathers, is treated with thioglycolic acid at a pH of 10 or higher the reaction appears to be identical with that on simpler disulfides. The reaction is a simple reduction; no loss of sulfur occurs. The sulfhydryl protein can be reoxidized to the disulfide state, and this disulfide protein is still soluble in acid or alkali and digestible by trypsin or pepsin.

The action of cyanide on wool is not quite so simple. A higher alkalinity is required (pH 12 to 13) and the proteins lose sulfur; however, the substances obtained behave as true proteins, not as products of hydrolysis.

The dissolving action of sodium sulfide has been known for a long time and is used industrially. Küster and coworkers (8) and Merrill (9) have considered the dissolving of keratin as a hydrolysis. Pulewka (10) and Speakman (11) realized that the action is on the disulfide groups. However, substances obtained behave as true proteins. In contrast to the cyanide preparations they contain more sulfur than the native keratin, though the cystine determinations by the Folin and Marenzi (12) method give the same values as the wool. It is likely that the action of Na_2S on keratins is not identical with its action on simpler disulfides and seems to be analogous to the action of sulfite on cystine, and a polysulfide is formed which behaves as cysteine in the Folin and Marenzi procedure. When such a protein is redissolved in weak alkali and reprecipitated with acid, it undergoes a loss in sulfur, but always in such a way that the total sulfur exceeds the cystine sulfur calculated from the cystine determination on the same preparation.

---

* Dr. A. E. Mirsky called our attention to the use of thioglycolic acid as a protein reductant. Unpublished work from this laboratory has shown that cystine is reduced at mildly alkaline reaction by an excess of thioglycolic acid. The cysteine has been almost quantitatively recovered by converting it to benzylcysteine.

† Unpublished work from this laboratory has shown that cystine is reduced according to Reaction 3. The cysteine and disodium-disulfide have been recovered as benzyl derivatives.

Sodium sulfite does not dissolve wool at an appreciable rate at any pH; so Reaction 4 has no practical significance for keratin.

EXPERIMENTAL

Most of the experiments were performed with native sheep wool, freed from fat by ether extraction. Other experiments with chicken feathers gave similar results. The wool was analyzed for cystine by the Folin and Marenzi (12) procedure, for nitrogen by the Kjeldahl method, and for sulfur by bomb combustion (13). The results, corrected for moisture, but not for ash, are as follows: N 16.66, S 3.19, cystine 11.90, 11.86 (calculated according to sulfur content, 11.9).

Protein Obtained with Thioglycolic Acid

50 gm. of wool were dissolved in 2 liters of a 0.5 M thioglycolate solution. This solution was prepared by neutralizing 92 gm. of thioglycolic acid with twice the amount of concentrated NaOH necessary to neutralize it to the turning point of phenol red, and made up to 2 liters. (The pH of the solution is about 12.) After 3 hours at 30° practically all the wool had dissolved. The mixture was filtered and the filtrate precipitated with 2 moles of glacial acetic acid, the precipitate collected on a filter, immediately twice ground with acetone made acid with HCl, washed with ether, and dried *in vacuo*.

The yield was 30 gm. of dry white powder which was still contaminated with a small amount of thioglycolic acid. The protein was freed of the last traces of thioglycolic acid by suspending in water and dialyzing for 3 days against distilled water in a cellophane tube. The fact that the protein was free from thioglycolic acid was shown by the cobalt test described in the appendix. The protein obtained is insoluble in water or neutral salts, but is soluble in strongly acid or alkaline solutions. The dialyzed proteins gave directly a strong nitroprusside test. They are slowly oxidized by air in bicarbonate solutions. Then the nitroprusside test of the protein is positive only after addition of KCN. The oxidized protein can be reduced over again by thioglycolic acid in bicarbonate buffers.

Four preparations have been made from wool by treatment for 1, 3, 6, and 24 hours with thioglycolate solutions. No increased yield is obtained after 3 hours. On varying the pH of the thioglycolate the wool does not dissolve at pH 9.0; it dissolves slowly at 10.1, and rapidly at a pH of 11 or over. Control experiments with M sodium carbonate or ammonium hydroxide showed that no dissolving action occurred in the absence of thioglycolate.

Analyses of two of the preparations are given below. The cystine determinations were made after first oxidizing the hydrolyzed protein with 3 per cent H_2O_2 (as Dr. E. A. Mirsky suggested to us) and then following the Folin-Marenzi method. The nitrogen determination was by micro-Dumas. The figures are in percentage of the dry weight and are not corrected for ash.

	Treated with thioglycolate for 24 hrs	Treated with thiogycolate for 3 hrs.
Sulfur	3.20	3.13
Nitrogen	15.71	15.75
Cystine	11.8	11.9
" calculated from sulfur	12.0	11.74
Isoelectric point	4.6	4.7

Proteins Prepared by Potassium Cyanide

Wool does not appreciably dissolve in 1 M KCN solutions, even in days, and in such a solution it turns dark brown to black. In 0.1 N NaOH and M or 0.5 M KCN most of the wool dissolves in a few hours, and such solutions do not become black even after days. If the wool is first treated with NaOH, washed, and exposed to KCN, it does not dissolve. The preparation of the protein from the solution was analogous to that described above.

A protein was prepared from wool by treating 50 gm. of wool with 2 liters of M KCN in 0.1 N NaOH. The yield was 17.6 gm. of dry protein. The protein is similar to that obtained by thioglycolic acid. It is slightly more soluble, lower in sulfur and cystine content. It gives the nitroprusside test and does not lose this property even when its alkaline solution is exposed to the air for days. Preparations made by treatment with KCN for 1, 6, 24, and 48 hours had sulfur contents of 1.3, 1.7, 1.8, and 1.4 respectively.

Analyses of two preparations are given below.

	Treated with KCN for 24 hrs.	Treated with KCN for 6 hrs.
Sulfur	1.8	1.73
Nitrogen	15.99	14.79
Cystine		1.86, 1.87
Isoelectric point	4.7	4.7

Preparation with Sodium Sulfide

50 gm. of wool were dissolved in 2 liters of 0.5 M Na_2S. The protein was isolated as above. The S content was very high, due in part to free sulfur extractable by CS_2. However, the final preparations gave high constant values of S, and this could not be decreased by further grinding with CS_2. When the entire preparation of the protein is carried out under nitrogen, no free sulfur is formed, but the sulfur content of the protein is still high. The protein so isolated gave a negative nitroprusside test (even when prepared under nitrogen) unless first treated with KCN. The isoelectric zone is wide, but one preparation, as colloidal solution made by dialyzing the Na_2S-wool solution without previous precipitation, gave a sharp isoelectric point at pH 4.9.

The analyses of two preparations are given below.

	Sample A. Treated with Na_2S for 48 hrs.	Sample B. Treated with Na_2S for 6 hrs.
Sulfur	6.61, 6.55	5.36
Nitrogen	12.99	13.62
Cystine	11.63	11.4
Isoelectric point	4.1–4.5	4.2–4.6

Sample A was redissolved in concentrated NH_4OH, filtered, and reprecipitated with acetic acid. On analysis the results obtained were: S, 3.93; cystine, 6.94 (cystine S, 1.85). Both the original and the reprecipitated protein, though differing in total S, contain approximately twice the amount of sulfur as corresponds to the cystine sulfur.

Solubility and Flocculation

All the proteins described above are insoluble in water and resemble casein or denatured proteins. The dry proteins can best be dissolved in weak alkalies by grinding in a mortar with Na_2CO_3 or $NaHCO_3$. In dilute NaOH they dissolve readily, but lose sulfur. When the filtrate of wool dissolved in Na_2S is dialyzed directly for several days, the protein remains in solution, but it is precipitated by a trace of acetic acid. Among the dry proteins, the cyanide preparation dissolves most readily.

The isoelectric points were estimated by the method of Michaelis and Rona (14). The dry proteins were ground with $NaCO_3$ and water, and filtered, so that the solution was 0.1 M in Na_2CO_3 and 0.5 per cent in protein. To a series of test-tubes 1 cc. of protein solution and increasing amounts of acetic acid were added, so calculated that all the tubes had a total volume of 10 cc. and an arithmetical series of pH differing by 0.3 pH. The optimum of flocculation was taken as the isoelectric point, and its pH and that of the adjoining tubes was determined with the glass electrode. The results obtained are tabulated among the analyses above. The isoelectric points for these protein preparations are slightly lower than the isoelectric point of native wool. Speakman (15) and Elöd and Silva (16) by independent methods obtained the values of 4.8 and 4.9.

Digestion by Trypsin and Pepsin

Trypsin, purified by the method of Anson and Mirsky (17), was added to suspensions of the proteins in bicarbonate buffers at pH of 8.5 to 8.8, and maintained at 37°. Samples were withdrawn and precipitated with sulfosalicylic acid. The controls showed complete precipitation, and practically all of the nitrogen was in the precipitate. The digestion series showed progressively less precipitation, and after 2 hours 90 to 95 per cent of the nitrogen was in the filtrate. All of the proteins described above are digested by trypsin, and the thioglycolic acid preparation is digested when it is either in the sulfhydryl or the disulfide state.

The dry proteins were suspended in dilute HCl, pepsin was added, and the mixture had a pH of 1.30. The suspensions, maintained at 37°, gradually became clear due to digestion by the pepsin. These solutions remained clear upon the addition of sulfosalicylic acid. Nitrogen determinations on the filtrates of control showed practically complete precipitation, and on the digestion series about 80 per cent digestion. All the proteins described above were similarly digested, and the state of the proteins as sulfhydryl or disulfide did not affect the result.

DISCUSSION

The keratins and fibroin are similar in their insolubility in the usual protein solvents, and in their indigestibility by pepsin and trypsin, but that they are essentially different in structure is shown by the agents which bring about their solution. Fibroin, though not soluble in water, dilute acids, or alkali, is easily dissolved in concentrated solutions of certain neutral salts such as calcium sulfocyanate or lithium iodide (18) and also in cold concentrated hydrochloric acid. These solutions can be easily reprecipitated, and the precipitate shows a fibrous structure. Wool cannot be dispersed[1] in any of those solvents that act on silk, but it can be readily dissolved in a series of agents that split the disulfide bond, as alkaline solutions of sodium cyanide, sulfide, and thioglycolate. These solutions do not dissolve silk in a perceptible degree. The protein, reprecipitated from a solution of wool in one of these agents, is never fibrous but amorphous.

These properties are in good agreement with the known differences in chemical composition and structure of these two classes of proteins. The x-ray diffraction studies of Brill (19) and Meyer and Mark (20) show that fibroin consists of fully extended polypeptide chains, and these chains must be oriented parallel to each other chiefly by residual forces for fibroin contains no cystine and but very little diamino and dicarboxylic acids. The regularity of the fully extended chain of amino acids in silk seems to be sufficient to bring about a tight fiber structure, without lateral chemical links, but this is not the case in the keratins. The x-ray studies of Astbury (21) on mammalian hairs have shown that these proteins are crystalline with partially contracted polypeptide chains. Speakman (22) has pointed out the importance of the lateral links between parallel polypeptide chains, the bond of the disulfide group of cystine, and the polar link of diamino and dicarboxylic acids. The stability of the keratin molecule depends on these bonds. Once these two types of bridges have been broken by chemical attack, the keratins behave in their solubility and digestibility by proteolytic enzymes as denatured proteins.

---

[1] Von Weimarn claims that wool can be dispersed in a concentrated boiling solution of calcium or lithium sulfocyanate at 180°. It is true that the wool dissolves very slowly, but the solution becomes alkaline and the presence of sulfides can be demonstrated. So the action can be best interpreted as a dissolution by alkaline sulfides formed from the slow decomposition of sulfocyanate.

The sulfhydryl protein, produced by the action of thioglycolic acid on wool, can be reoxidized to the disulfide state by air, in moderately alkaline solution, or by hydrogen peroxide. The reoxidized protein, in spite of its disulfide bonds, is still soluble in alkali, is enzyme-digestible, and has about the same isoelectric point. In the reoxidation of the sulfhydryl protein, the original crystalline pattern is not reformed, the disulfide bonds are no longer rungs of a ladder connecting parallel polypeptide chains. This shows that though the disulfide bond is essential for the properties of native keratin, these properties cannot be ascribed to the purely chemical character of this group. It is the physical pattern of the keratin, with a definite spatial arrangement of peptide chains and disulfide bonds, which imparts to keratin its resistance to enzyme hydrolysis and dissolving agents.

Mention should be made of the fact that all of the agents used to dissolve wool require a higher alkalinity than to reduce a simple disulfide such as cystine. The pH used is never high enough to dissolve wool of its own accord in a comparable time, yet the necessity of a distinctly alkaline reaction is striking and demands an explanation.

The following hypothesis may be offered. The cross links between peptide chains in keratin are of two kinds, disulfide links and bridges formed by the electrostatic attraction of the NH_3^+ group of the diamino acids for the COO^- group of the dicarboxylic acids. These salt-like bridges will be broken in alkaline solution by removal of a proton from the amino group. It appears that it is necessary to open these links before the disulfide groups may be reduced.

SUMMARY

Keratin dissolves in Na_2S, KCN, or thioglycolic acid at alkaline reaction. This effect is chiefly due to the splitting of the disulfide groups, which are essential for the maintenance of the fibrous structure of keratin. Chemically, the action of thioglycolic acid is the simplest; it simply reduces the disulfide to sulfhydryl groups with no other appreciable chemical change. The other reagents act in a more complicated way. The substances thus obtained are proteins. They are soluble in alkali or acid, with a definite isoelectric point, and they are digestible by pepsin and trypsin, even when secondarily the —SH group has been reoxidized to the —SS— stage, or when due to secondary reactions the sulfur content has been greatly changed.

Appendix

Test for Thioglycolic Acid—In order to test whether the protein prepared with thioglycolic acid is completely freed from this acid the following test was applied. A suspension of the protein in sodium pyrophosphate solution is mixed with a drop of a 1 per cent solution of cobalt sulfate. Any trace of free thioglycolic acid will develop, either immediately or after some time, on exposing the mixture to the air, a brown color. The sulfhydryl protein does not give this test, although it gives a positive nitroprusside test which is common for all sulfhydryl compounds. This test is based on the formation of strongly colored cobalti complexes of thioglycolic acid according to Michaelis and Schubert (23). The smallest amount of thioglycolic acid giving a distinct positive test in a volume of 1 cc. is approximately 0.05 mg. The oxidized form of thioglycolic acid, dithiodiglycolic acid, can be tested for with the same reagent, adding besides some Na_2SO_3 which reduces the disulfide to the sulfhydryl compound.

BIBLIOGRAPHY

1. Lissizin, T., *Z. physiol. Chem.*, **173**, 309 (1928).
2. Stary, Z., *Z. physiol. Chem.*, **136**, 160 (1924); **144**, 147 (1925); **175**, 178 (1928).
3. Waldschmidt-Leitz, E., and Schuckmann, G., *Ber. chem. Ges.*, **62**, 1891 (1929).
4. Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).
5. Mauthner, J., *Z. physiol. Chem.*, **78**, 28 (1912).

6. Anson, M. L., and Mirsky, A. E., *Proc. Soc. Exp. Biol. and Med.*, **28**, 170 (1930).
7. Clarke, H. T., *J. Biol. Chem.*, **97**, 235 (1932).
8. Küster, W., Kumpf, W., and Köppel, W., *Z. physiol. Chem.*, **171**, 114 (1927).
9. Merrill, H. B., *Ind. and Eng. Chem.*, **17**, 36 (1925).
10. Pulewka, P., *Z. physiol. Chem.*, **146**, 130 (1925).
11. Speakman, J. B., *Proc. Roy. Soc. London, Series A*, **132**, 167 (1931).
12. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
13. Elek, A., and Hill, D. W., *J. Am. Chem. Soc.*, **55**, 3479 (1933).
14. Michaelis, L., and Rona, P., *Biochem. Z.*, **27**, 38 (1910).
15. Speakman, J. B., *J. Soc. Dyers and Colourists*, **41**, 172 (1925).
16. Elöd, E., and Silva, E., *Z. physik. Chem.*, **137**, 142 (1928).
17. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **17**, 151 (1933).
18. von Weimarn, P. P., in Alexander, J., Colloid chemistry, New York, **4**, 363 (1932).
19. Brill, R., *Ann. Chem.*, **434**, 204 (1923).
20. Meyer, K. H., and Mark, H., *Ber. chem. Ges.*, **61**, 1932 (1928).
21. Astbury, W. T., *Tr. Faraday Soc.*, **29**, 193 (1933); Fundamentals of fiber structure, London (1933).
22. Speakman, J. B., and Hirst, M. C., *Tr. Faraday Soc.*, **29**, 148 (1933).
23. Michaelis, L., and Schubert, M. P., *J. Am. Chem. Soc.*, **52**, 4418 (1930).

TRYPTOPHANE METABOLISM

VII. GROWTH AND KYNURENIC ACID PRODUCTION ON AMIDES OF *l*-TRYPTOPHANE*

By LYLE C. BAUGUESS AND CLARENCE P. BERG

(*From the Biochemical Laboratory, State University of Iowa, Iowa City*)

(Received for publication, May 21, 1934)

Comparisons between the metabolism of free amino acids and the metabolism of amino acids in which either the amino or the carboxyl group has been blocked by a substituent radical provide information concerning the nature and specificity of body cleavages. Most studies of this type recorded in the literature have considered only derivatives in which the radical is substituted in the α-amino group. A few metabolism studies have been made on amino acid esters. Berg, Rose, and Marvel (1929–30) reported that the nutritive value of *l*-tryptophane is not impaired by esterification with ethyl alcohol. Likewise, the benzyl and phenyl ester hydrochlorides of *l*-tryptophane (Berg and Hanson, unpublished data) effectively replace *l*-tryptophane in supporting growth when fed in conjunction with a tryptophane-deficient basal diet. Also in the case of the ethyl ester hydrochloride of *l*-tryptophane, the substituent radical does not prevent the production of kynurenic acid in the rabbit (Berg, 1931).

So far as we are aware, no similar experiments on amino acid amides have been made. Numerous *in vitro* studies (*e.g.* Balls and Köhler, 1930–31) indicate that peptide-splitting enzymes vary considerably in specificity, some apparently requiring the presence of a free amino group in the peptide substrate, others the presence of a free carboxyl group, and still others neither a free amino nor a free carboxyl group. Balls and Köhler (1930–31, *a*) have shown that the cleavage of "aniline peptides" by intestinal erepsin is influenced by substituent radicals in the aromatic nucleus, apparently because of their effect on the acid character of the imide group of the peptide linkage which they believe to be a point of attachment for all peptidases (1930–31, *b*). Bergmann, Zervas, Schleich, and Leinert (1932) have found that *d*-alanyl-*l*-proline and glycyl-*l*-proline, which may be regarded as tertiary amides of alanine and glycine, can be split by the aminopolypeptidase, but not by the proteinase or dipeptidase fractions of erepsin. These are only a few examples from a host of experiments bearing upon the effect of chemical structure on *in vitro* enzymolysis.

---

* A portion of this communication was presented before the American Society of Biological Chemists at New York, March 30, 1934 (*J. Biol. Chem.*, **105**, viii (1934)).

The experimental data are taken from a dissertation submitted by Lyle C. Bauguess in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

The study of the cleavage of free and substituted amino acid amides in the animal body is essentially a study of the collective ability of the enzymes coming in contact with such amides under body conditions, to hydrolyze them. The availability of an amide of tryptophane as a supplement in a tryptophane-deficient diet for growth purposes in the rat would imply the ability of that animal to hydrolyze the amide. The formation of kynurenic acid in the rabbit would possess the advantage of indicating whether large quantities of the compound could be split readily; subcutaneous administration in such studies would serve to exclude the action of the enzymes of the alimentary tract. Accordingly, we have used both growth and kynurenic acid production as criteria in studying *in vivo* cleavage of the unsubstituted and the ethyl- and diethylamides, as well as of the unsubstituted and the N-ethylanilides of tryptophane.

EXPERIMENTAL

The compounds used were all prepared in this laboratory. The tryptophane was obtained from casein according to the method of Cox and King (1930). It was converted to the amide by preparing the hydrochloride of tryptophyl chloride, essentially as directed by Abderhalden and Kempe (1927), and treating the latter with the appropriate amine as outlined below.

The hydrochloride of tryptophyl chloride melted at 230° (uncorrected) with gas evolution. Analysis showed 27.05 per cent chlorine. Abderhalden and Kempe report 228° and 26.71 per cent, respectively. The theoretical chlorine content is 27.38 per cent.

In the synthesis of the amides, 0.04 mole of the hydrochloride of tryptophyl chloride (10.36 gm.) was suspended in 80 cc. of anhydrous ether or chloroform in a reaction vessel cooled in an ice-salt bath. 0.12 mole of the appropriate amine was added slowly and with vigorous stirring, after which the reaction flask was stoppered and placed in the refrigerator overnight. The reaction mixture was then concentrated *in vacuo*, the residue dissolved in a minimum amount of hot methyl alcohol, and treated with boneblack three times. The chloride content of the methyl alcohol solution was determined and the calculated amount of 2 N NaOH added to neutralize the HCl. The bulk of the NaCl was removed by concentrating to dryness *in vacuo*, dissolving the major portion of the residue in hot absolute methyl alcohol, and filtering. The product was further purified through the mercury salt. Sufficient 1:3 H_2SO_4 was added to the methyl alcohol solution to make the concentration of H_2SO_4 7 per cent by volume. This was followed by the addition of 500 cc. of a 10 per cent solution of mercuric sulfate containing 7 per cent of sulfuric acid. The yellow precipitate was filtered and washed free of chlorides, with a 1 per cent solution of mercuric sulfate in 5 per cent sulfuric acid, and of excess H_2SO_4, with water. The free amide was obtained from the mercuric sulfate complex by suspending the latter in water, rendering alkaline to phenolphthalein with a hot saturated solution of barium hydroxide, and decomposing by passing in a stream of hydrogen sulfide while stirring. The mercuric sulfide-barium sulfate precipitate was filtered off, washed twice with small portions of hot water, and discarded. The combined washings and filtrate were freed of barium ion by the careful addition of cold dilute H_2SO_4, the $BaSO_4$ removed by filtration, and the filtrate concentrated to dryness *in vacuo*. The residue represented the free amide. It was again taken up in absolute methyl alcohol, filtered, and the filtrate evaporated to dryness.

The properties of the several amides thus prepared are summarized in Table I, together with those of the tryptophane used.

The average yields varied from 48.9 per cent of the calculated amount for tryptophaneethylamide to 72.0 for the tryptophaneethylanilide. All of the amides were soluble in water and methyl and ethyl alcohol. They responded to the Hopkins-Cole, but not to the bromine water, test for tryptophane.

Growth Studies—The compounds were tested from the standpoint of growth and kynurenic acid production. In the assay for growth-promoting ability, they were incorporated in a tryptophane-deficient basal diet which consisted of acid-hydrolyzed casein (see Berg and Rose, 1929) 14.7, cystine 0.3, starch 39.5, sucrose 15, Crisco 19, cod liver oil 5, salt mixture (Hawk and Oser, 1931) 4.5, and agar 2 per cent. The tryptophane or amide supplement supplanted an equal weight of hydrolyzed casein. The diets were fed *ad libitum*. Vitamin B complex was supplied daily in the form of pills containing 200 mg. of yeast[1] and 100 mg. of starch.

TABLE I

Physical and Chemical Constants of l-Tryptophane and l-Tryptophaneamides

Compound	Melting point (uncorrected)	$[\alpha]_D^{20}$ (2 per cent solution in ethyl alcohol)	Nitrogen	
			Found	Calculated
	°C.		*per cent*	*per cent*
l-Tryptophane	278	−33.4*	13.70	13.72
Tryptophaneamide	167–170	−7.9	20.54	20.69
Tryptophanemonoethylamide	67–69	−14.4	18.14	18.18
Tryptophanediethylamide	183–185	−24.7	16.22	16.21
Tryptophaneanilide	83–85	−9.5	15.00	15.05
Tryptophaneethylanilide	97–99	−4.2	13.69	13.68

* 0.5 per cent solution in water.

Five litter mate rats were used in the test on each compound, two serving as control, and three as experimental animals. One rat received a diet containing 0.2 per cent of tryptophane, a second animal received no tryptophane, and the other three received the appropriate amide in an amount equivalent to 0.2 per cent of tryptophane.

Representative growth curves of the experimental and of the control animals are shown in Chart I, individual growth and food consumption data in Table II. A comparison of growth rates and food consumption of the rats receiving the amides with those of the rats fed tryptophane reveals no significant differences.

---

[1] The yeast was kindly supplied by the Northwestern Yeast Company, Chicago.

Kynurenic Acid Studies—The technique employed in testing the tryptophane amides for kynurenic acid production has been discussed elsewhere (Berg, 1931) in detail. Briefly, each of the amides was administered to male rabbits (either orally or subcutaneously) as the hydrochloride, and the kynurenic acid was isolated from the urine of the following 24 hours according to the

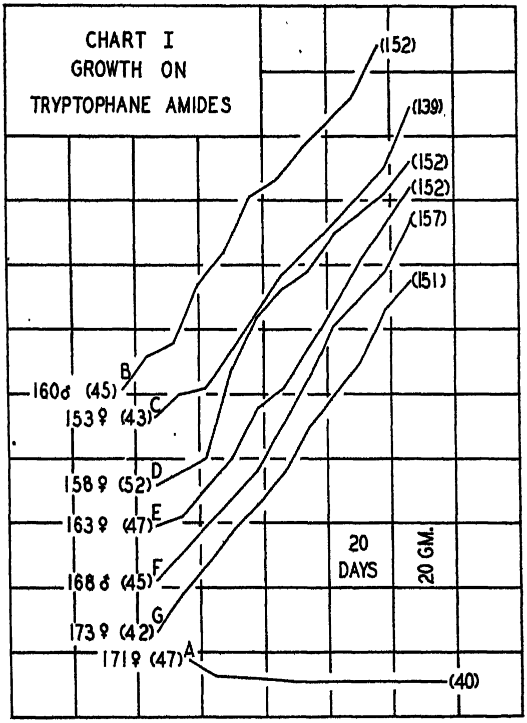


CHART I. Each curve represents the growth of one of three or more animals receiving the same diet. Curve A represents no supplement; Curve B, *l*-tryptophane, 0.2 per cent; Curve C, *l*-tryptophaneamide, 0.199 per cent; Curve D, *l*-tryptophaneethylamide, 0.226 per cent; Curve E, *l*-tryptophanediethylamide, 0.254 per cent; Curve F, *l*-tryptophaneanilide, 0.269 per cent; and Curve G, *l*-tryptophaneethylanilide, 0.301 per cent. Initial and final weights are given in parentheses.

Capaldi (1897) procedure. The kynurenic acid precipitate was washed with water-saturated butyl alcohol to remove any coprecipitated derivative. During the period of study, the rabbits were housed in individual metabolism cages and given water and oats *ad libitum*. The compounds were administered every 3rd day. All 24 hour urines, whether collected for control or experimental periods, were analyzed according to the routine procedure.

TABLE II

Food Consumption and Body Weight Changes on a Tryptophane-Deficient Diet Supplemented with Tryptophaneamides

Rat No. and sex	Average daily		Total change in weight	Supplement*
	Change in weight	Food consumption		
	gm.	*gm.*	*gm.*	
155 ♀	+1.19	7.9	+95	Tryptophane
152 ♀	+0.98	7.9	+78	Tryptophaneamide
153 ♀	+1.20	7.8	+96	"
154 ♂	+1.23	7.8	+98	"
151 ♂ †	−0.33	3.0	−8	None
160 ♂	+1.34	8.0	+107	Tryptophane
157 ♂	+1.23	7.9	+98	Tryptophaneethylamide
158 ♀	+1.25	7.6	+100	"
159 ♂	+1.28	7.7	+102	"
156 ♂	−0.16	3.0	−13	None
165 ♂	+1.20	7.4	+96	Tryptophane
162 ♂	+1.36	7.5	+109	Tryptophanediethylamide
163 ♀	+1.31	7.8	+105	"
164 ♀	+1.30	7.7	+104	"
161 ♀	−0.13	2.8	−10	None
170 ♀	+1.45	7.7	+116	Tryptophane
167 ♀	+1.44	7.6	+115	Tryptophaneanilide
168 ♂	+1.40	7.8	+112	"
169 ♀	+1.33	7.6	+106	"
166 ♂	−0.04	2.7	−3	None
175 ♂	+1.45	7.2	+116	Tryptophane
172 ♂	+1.44	7.3	+115	Tryptophaneethylanilide
173 ♀	+1.36	7.3	+109	"
174 ♂	+1.28	7.4	+102	"
171 ♀	−0.09	2.7	−7	None

* All supplements were equivalent to 0.2 per cent of tryptophane.

† Rat 151 died at the end of 24 days.

The results of the experiments appear in Tables III and IV. The amounts of precipitate obtained following the oral or subcutaneous administration of the tryptophane derivatives were not significantly different from those secured after the administration of the free *l*-tryptophane in equivalent amount. Without exception, the precipitates melted between 250–252° and 268–272° (uncorrected) and showed melting points intermediate between these and that of partially purified kynurenic acid (m.p. 275°) when previously mixed with the latter. The Jaffe (1882–83) test was positive in each case. Kynurenic acid, isolated under similar conditions following tryptophane administration, usually melts in the same range (around 260°, uncorrected). There can be little doubt, therefore, that the substance isolated was kynurenic acid. Hopkins-Cole tests on the urines following the subcutaneous administration of the amides were all negative, indicating complete catabolism of the compounds. After oral administration,

TABLE III

Kynurenic Acid Elimination Following Subcutaneous Administration of Tryptophaneamides

Day	Kynurenic acid precipitate after washing with 5 cc. butyl alcohol		*l*-Tryptophane or amide (administered in 2 equal doses 9 hrs. apart, as hydrochloride)*
	Rabbit 5 ♂, 2.5 kilos	Rabbit 6 ♂, 2.6 kilos	
	gm.	*gm.*	
1–2	0.0027	0.0023	
3	0.2406	0.2576	1 gm. of *l*-tryptophane
4–5	0.0021	0.0026	
6	0.2303	0.1712	0.995 gm. of tryptophaneamide
7–8	0.0030	0.0019	
9	0.2501	0.2763	1.132 " " tryptophanemonoethylamide
10–11	0.0023	0.0019	
12	0.2671	0.1848	1.270 " " tryptophanediethylamide
13–14	0.0013	0.0016	
15	0.2099	0.2348	1.505 " " tryptophaneethylanilide
16–17	0.0049	0.0058	
18	0.2670	0.2341	1.367 " " tryptophaneanilide
19–20	0.0038	0.0006	

* Each dose was equivalent to 0.5 gm. of tryptophane.

the tests were positive. Since the slower absorption following oral administration should favor, rather than hinder, the metabolism of the amides, one can hardly ascribe the color produced to them or to tryptophane. It must be due, rather, to other indole derivatives produced from them by intestinal bacteria, substances which are absorbed, but being catabolized with difficulty, are consequently excreted in the same or in some modified form.

Table IV

Kynurenic Acid Elimination Following Administration of Tryptophaneamides by Stomach Tube

Day	Kynurenic acid precipitate after washing with 5 cc. butyl alcohol		*l*-Tryptophane or amide (administered in 2 equal doses, 9 hrs. apart, as hydrochloride)*
	Rabbit 7 ♂, 2.7 kilos	Rabbit 8 ♂, 2.6 kilos	
	gm.	*gm.*	
1–2	0.0028	0.0041	
3	0.2474	0.2020	1 gm. of *l*-tryptophane
4–5	0.0035	0.0064	
6	0.2833	0.2142	0.995 gm. of tryptophaneamide
7–8	0.0018	0.0027	
9	0.2485	0.1639	1.132 “ “ tryptophanemonoethylamide
10–11	0.0011	0.0023	
12	0.2341	0.2009	1.270 “ “ tryptophanediethylamide
13–14	0.0027	0.0018	
15	0.2419	0.1645	1.505 “ “ tryptophaneethylanilide
16–17	0.0039	0.0039	
18	0.2009	0.2178	1.367 “ “ tryptophaneanilide
19–20	0.0020	0.0043	

* Each dose was equivalent to 0.5 gm. of tryptophane.

Obviously, from the growth and the kynurenic acid studies, all of the amides undergo ready cleavage in the animal organism. In the rabbit, such cleavage is as readily effected when the compound is introduced subcutaneously as when it is given orally.

SUMMARY

The free amide, the ethylamide, and the diethylamide, as well as the free anilide and the N-ethylanilide, of tryptophane have been prepared and tested for *in vivo* cleavage by feeding them to rats as supplements in a diet deficient in tryptophane, and by testing them for kynurenic acid production in the rabbit. All readily support growth and undergo conversion to kynurenic acid. Apparently the primary, secondary, or tertiary nature of the amide does not of itself influence the cleavage of the amide *in vivo*.

BIBLIOGRAPHY

Abderhalden, E., and Kempe, M., *Z. physiol. Chem.*, **52**, 207 (1927).
Balls, A. K., and Köhler, F., *Ber. chem. Ges.*, **64**, 34 (1930–31, *a*); **64**, 294 (1930–31, *b*); **64**, 383 (1930–31, *c*).
Berg, C. P., *J. Biol. Chem.*, **91**, 513 (1931).
Berg, C. P., and Hanson, H. E., unpublished data.
Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, **82**, 480 (1929).
Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 207 (1929–30).
Bergmann, M., Zervas, L., Schleich, H., and Leinert, F., *Z. physiol. Chem.*, **212**, 72 (1932).
Capaldi, A., *Z. physiol. Chem.*, **23**, 92 (1897).
Cox, G. J., and King, H., in Clarke, H. T., *et al.*, Organic syntheses, New York, **10**, 100 (1930).
Hawk, P. B., and Oser, B. L., *Science*, **74**, 369 (1931).
Jaffe, M., *Z. physiol. Chem.*, **7**, 399 (1882–83).

EXTRACTION STUDIES ON THE ADRENAL CORTICAL HORMONE

I. METHODS OF PREPARATION*

By J. J. PFIFFNER, HARRY M. VARS, and A. R. TAYLOR

(*From the Biological Laboratory, Princeton University, Princeton*)

(Received for publication, June 13, 1934)

A method of extraction for the adrenal cortical hormone was described some time ago (1, 2). In this early work the length of the survival period of the adrenalectomized cat served as the biological criterion of physiological potency. Some further observations on extraction technique and chemical properties of the hormone were made, the relief of the symptoms of adrenal insufficiency in the same species being used as a guide (3). Neither of these biological criteria was satisfactory from a chemical standpoint, since the results obtained had only a qualitative significance. The development of a method of assay based upon the minimum maintenance requirement of the adrenalectomized dog (4) made it possible to obtain quantitative information concerning the preparation of the hormone. These quantitative data are summarized in this report.

Method of Assay—The dog method of assay was used (4). Details of the technique as employed in this laboratory have been reported (5). The animals were standardized against a single

---

* This investigation was supported by a grant from the Josiah Macy, Jr., Foundation.

We wish to express our appreciation to the management and research staff of Parke, Davis and Company for their generous cooperation.

An abstract of a portion of these data was read before the American Society of Biological Chemists at Philadelphia, April 28–30, 1932 (Pfiffner, J. J., Vars, H. M., Bott, P. A., and Swingle, W. W., *J. Biol. Chem.*, **100**, lxxviii (1933)).

TABLE I

Yield of Cortical Hormone Obtained from Beef Adrenal Glands

Experiment No.	Starting material and method of extraction*	Yield per kilo tissue			Dog units per mg. active fraction
		Assay results	Average	Fraction weight	
		dog units	*dog units*	*mg.*	
1	Dissected cortex	>300 <400	350	80	4
2	" "	>2640 <3300	2870	73	39
3-a	" "	>3330 <5500	4400	83	53
3-b	Medulla	<500	<500	80	<6
3-c	Whole gland		3330†		
4	" " ‡	>2000 <2900	2500	59§	42
5	Chilled and packed in ice 24 hrs.	>1700 <2700	2200	59	37
6	Frozen 30 days at −5°	>1500 <3000	2250	60	37
7	" 6 mos. " −5°	>1000 <2000	1500	63	24
8	" and packed in dry ice 3 days	>2000 <3000	2500	55	45
9	Autolyzed 48 hrs. at room temperature (23°)	>1000 <2000	1500	42	36
10‖	Ethyl alcohol	>1700 <2700	2200	59	37
11‖	" " + 0.25% acetic acid	>1000 <2000	1500	80	19
12‖	Ethyl alcohol + 0.5% acetic acid	<1000	<1000	133	<8
13‖	Ethyl alcohol made 0.08 N with H_2SO_4	>380 <750	615	242	<3
14	Ethyl alcohol made 0.08 N with H_2SO_4 (neutralized before distillation)	>750 <1500	1125	38	30
15	Kutz (6)	>125 <250	187	200§	<1
16	Kendall *et al.* (7, 8)	>750 <1500	1125	395§	<3
17	Gland residue from standard method extracted by Kendall's method	<100	<100	88	<2
18	Aqueous residue from standard method extracted by Kendall's method	>50 <100	75	106	<1
19	Neutral acetone¶	>1000 <2000	1500	100	15
20	Grollman and Firor (9)	>500 <1000	750	160§	<5

---

* 4 kilos of glands were used as the starting material in each experiment. Our standard method of extraction referred to in the text was used with the modifications or exceptions noted.

TABLE I—*Concluded*

† Calculated from the yields and relative weights of cortex (73 per cent) and medulla (27 per cent).

‡ The results recorded are the averages obtained from three experiments in which glands collected at various times during a 6 month interval were used. The respective average yields were 2250, 2400, and 2750 dog units. The fraction weight ranged from 50 to 67 mg.

§ The active fractions obtained in Experiments 4, 15, 16, and 20 from 1 kilo of whole beef adrenals contained 0.01, 0.10, 0.02, and 0.10 mg. of adrenalin, respectively. The adrenalin was determined by bioassay (blood pressure reaction in anesthetized dog).

|| The yields of adrenalin obtained in Experiments 10, 11, 12, and 13 were as follows: 0.14, 0.18, 0.19, and 0.22 per cent.

¶ Tissue extracted for 24 hours with 2.5 volumes of acetone; extractives fractionated by standard method.

preparation of hormone. The error of determination under the conditions employed is approximately ±25 per cent.

Source of Glands—Beef adrenals were used. In the early work the glands were received in the laboratory packed in ice on the day following collection at the abattoir. Later the glands were collected at the abattoir for a period of several days, frozen, and shipped to the laboratory packed in dry ice.

Methods of Extraction—4 kilos of glands served as the starting material in each experiment with the exception of those in which dissected cortex or medulla was employed. 3 kilos of dissected cortex and 1 of dissected medulla were used. The method of extraction employed unless otherwise specified was that described earlier (2, 3).

Relative Yield from Cortex, Medulla, and Whole Gland—The first assays of dissected cortex extract prepared in this laboratory were carried out at the Johns Hopkins Hospital by Drs. Harrop and Weinstein. Their assays showed a yield of 200 to 300 dog units per kilo of cortex (4). When assay work was started in this laboratory in the fall of 1931, their results were confirmed (Experiment 1, Table I). Further work showed that whole gland extracts had from 5 to 10 times this activity, which seemed to indicate the possible presence of a high concentration of hormone in the medulla. In Experiment 3, 4 kilos of whole gland were dissected and the medulla and cortex extracted separately and assayed. In this, as well as in all later experiments, dissected cortex yielded as much hormone as whole gland.[1] We are unable at the present time to offer an explanation for the differences in yield obtained from dissected cortex. Both the technique of extraction and assay were, so far as we are aware, held constant.

No significant variation could be detected in the yield of hormone obtained from glands collected during the various seasons of the year.

Effect of Previous Handling of Glands—The necessity of great precaution in handling the adrenal glands prior to extraction has been emphasized by various workers. Data are presented on the yield obtained from glands handled in a variety of ways. Glands can be frozen for prolonged periods without an appreciable effect on the yield. Tissue frozen for 6 months assayed 1500 dog units as compared to 2200 dog units for recently collected material. The good yield of hormone from autolyzed material is particularly interesting in view of the extensive precautions taken by Grollman and Firor (9) to avoid the destructive influence of enzymatic processes in the cell.

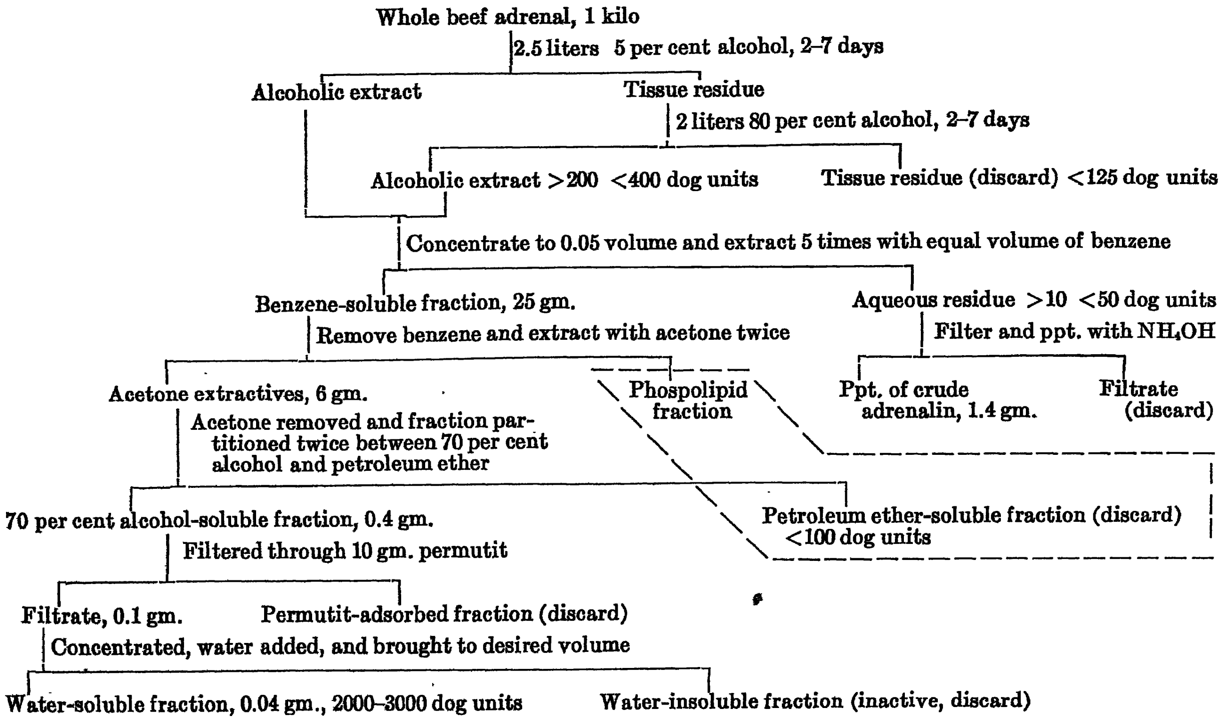
Desiccated whole adrenal gland has long been used orally in the treatment of various types of adrenal deficiency. The beneficial effect observed by many investigators must have been due to some substance other than the cortical hormone, since the yield of hormone from whole adrenal glands desiccated at a low temperature is less than 250 dog units per kilo of fresh whole gland.

Efficiency of Extraction Procedure—The magnitude of the losses encountered in the various steps is presented in the accompanying diagram. The major portion of the hormone is readily extracted

---

[1] A sample of the whole gland extract prepared in Experiment 4 (Table I) was submitted to Dr. G. A. Harrop of the Johns Hopkins Hospital for assay in March, 1932. He reported (private communication) a yield of approximately 2000 dog units per kilo of gland. A sample was also submitted to Drs. Oliver Kamm and N. North of the Research Laboratories of Parke, Davis and Company for assay on their colony of adrenalectomized dogs. They found the yield to be greater than 1000 dog units per kilo of gland but did not carry the assay further (private communication). Hence the high yield of hormone obtained by whole gland extraction was confirmed on three different groups of adrenalectomized dogs in as many laboratories. In every instance the yield was found to be many times (5 to 10) greater than that obtained previously from dissected cortex.

Flaw Sheet of Fractionation Procedure for Preparation of Adrenal Cortical Hormone with Fraction Weights and Losses Encountered at Each Step


Whole beef adrenal, 1 kilo
2.5 liters 5 per cent alcohol, 2–7 days
Alcoholic extract
Tissue residue
2 liters 80 per cent alcohol, 2–7 days
Alcoholic extract >200 <400 dog units
Tissue residue (discard) <125 dog units
Concentrate to 0.05 volume and extract 5 times with equal volume of benzene
Benzene-soluble fraction, 25 gm.
Aqueous residue >10 <50 dog units
Remove benzene and extract with acetone twice
Filter and ppt. with NH₄OH
Acetone extractives, 6 gm.
Phospholipid fraction
Ppt. of crude adrenalin, 1.4 gm.
Filtrate (discard)
Acetone removed and fraction partitioned twice between 70 per cent alcohol and petroleum ether
70 per cent alcohol-soluble fraction, 0.4 gm.
Petroleum ether-soluble fraction (discard) <100 dog units
Filtered through 10 gm. permutit
Filtrate, 0.1 gm.
Permutit-adsorbed fraction (discard)
Concentrated, water added, and brought to desired volume
Water-soluble fraction, 0.04 gm., 2000–3000 dog units
Water-insoluble fraction (inactive, discard)

On [illegible] Vol. 106, No. 2, September, 1934, line 4, read 2 [illegible] per cent [illegible] days for 2.5 liters 5 per cent alcohol [illegible].

with a single alcohol treatment. An additional 300 dog units can be obtained with a second alcohol extraction. A negligible amount of hormone remains in the gland tissue after two such extractions. The alcohol extracts are concentrated about 20 times. The exact degree of concentration is of no particular consequence, since the hormone is readily extracted from the concentrate with benzene, even though as much as 20 per cent of alcohol is present in the concentrate. On extracting the concentrate with benzene, emulsions are formed which break slowly. This emulsion formation is an advantage, since the interface of the two phases is increased tremendously over periods of hours without repeated shaking. As the benzene extraction proceeds, the tendency to form emulsions becomes less. 8 to 12 hours are allowed for the separation of the two phases. After five benzene extractions very little if any emulsion remains. The aqueous residue, including the small amount of emulsion, retains only about 30 dog units of cortical hormone. All of the hormone is readily extracted from the benzene-soluble fraction with two acetone treatments; the first acetone extraction is carried on by rubbing and allowing the material to stand overnight in the refrigerator. The lipid mass becomes friable and can be transferred readily to a mortar for the second extraction. This step effects a 4-fold concentration of activity. In the early work the acetone-soluble fraction was distributed four times between 70 per cent alcohol and petroleum ether. Less than 100 dog units are lost in two distributions, with proper technique. The activity is concentrated about 15 times. A 4-fold concentration is effected by permutit filtration with no loss of hormone. The final water-soluble fraction constitutes about 50 per cent of the fraction passing through the permutit filter. It varies considerably in weight in different preparations, ranging from 30 to 60 mg. per kilo of gland. This depends upon the length of time taken for the original alcohol extraction.

The mechanical losses in the various fractionation steps total about 300 dog units, with a yield of 2000 to 3000 dog units per kilo. 75 per cent of hormone added to inactivated adrenal material can be recovered. Recovery experiments were carried out as follows: 4 kilos of glands were extracted twice with alcohol as usual. The alcohol extracts were fractionated as indicated in the flow sheet. All the discarded fractions were saved, combined, and inactivated in a boiling water bath for 5 hours. This inactivated material was added to the thoroughly extracted tissue. To this gland mixture, which represented adrenal starting material minus the cortical hormone as closely as it could be reproduced, was added a definite number of dog units of cortical hormone and the mixture subjected to the complete fractionation procedure. In the first experiment 176 mg. of a fraction containing 6800 dog units were added and a fraction weighing 160 mg. and containing 5000 dog units recovered. In the second experiment 8000 dog units were added and 6000 dog units recovered.

Stability of Hormone in Various Stages of Extraction—Considerable emphasis has been laid by some workers on the necessity of very rapid extraction of the hormone from the gland. Ground glands can be stored in 2.5 volumes of 95 per cent alcohol for 3 weeks at room temperature with no appreciable loss in activity. However, the yield is less than 500 dog units after a 10 week period. The benzene-soluble fraction can be stored at room temperature for 11 weeks without loss of activity. The permutit-purified fractions preserved in 95 per cent ethyl alcohol at 6° for 2½ years still retain their initial activity. The finished aqueous extracts, if preserved with 0.1 per cent benzoic acid,[2] are equally stable. The pH of these aqueous solutions is usually between 4.0 and 5.5.[3]

In the several fractionation steps involving concentration of relatively large volumes such concentration has been effected by distillation under reduced pressure at an external bath temperature of 45–55°. The permutit-purified fraction can be refluxed in 95 per cent alcohol for 20 minutes without loss of activity, while no deterioration occurs in aqueous solutions of the permutit-purified fraction when held at 80° for the same length of time.

Influence of Acid Extraction on Yield—The effect of acid was studied in an effort to increase the yield of adrenalin reclaimable as a by-product. The results obtained with acetic and sulfuric acids are summarized in Experiments 10 to 14 (Table I). The addition of acetic acid affected the yield of adrenalin favorably but had the opposite effect on the yield of cortical hormone. 0.5 per cent acetic acid decreased the yield of cortical hormone by more than 50 per cent. The amount of impurities present in the final extract was increased markedly. After the completion of the foregoing experiments with acetic acid, Kendall *et al.* (7, 8) stated that sulfuric acid-acetone extraction increased the yield of cortical hormone. Experiments 13 and 14 show that the yield is decreased with sulfuric acid-alcohol extraction, either with or without neutralization before concentration (see p. 633).

---

[2] The use of benzoic acid as a preservative for aqueous solutions of the hormone was suggested by Dr. Oliver Kamm.

[3] In preparing the permutit-purified fraction for injection it is preferable to add the sodium chloride and benzoic acid before subjecting the suspension to Seitz filtration. The reduction in pH in the presence of sodium chloride causes the suspended material to coalesce, thereby increasing the rate of filtration. Cleghorn (10) observed a similar effect.

Other Methods of Extraction—Several methods for the preparation of the cortical hormone have been described (6–9, 11–17). Some procedures were suggested on the grounds of increased yield and greater purity of product. Fractionation steps were employed which in our work are shown to be inefficient. Therefore, the yields obtained with several of the proposed methods were compared under standardized assay conditions.

Hartman and Brownell (11) extracted dissected cortex with ether. The ether-soluble fraction was purified by freezing inactive material from various concentrations of ethyl alcohol and water. No specific fractionation step was used for the separation of adrenalin from the cortical hormone. Their method is inadequate for the preparation of extract from *whole* beef adrenal gland because of the toxic substances present in the medulla (15, 16).

A simplified method of extraction was suggested by Kutz (6). Ground beef adrenal glands were extracted with acetone. The aqueous sludge obtained on concentration of the acetone extract was extracted with benzene. The concentrated benzene washes were freed of adrenalin by washing with 4 per cent $NaHCO_3$. The benzene-soluble fraction was suspended in water and the water-insoluble material removed by filtration. This method gave a yield of 200 dog units per kilo of gland (Experiment 15). The hormone is readily extracted from the gland with acetone, as demonstrated in Experiment 19. In this process the major share of the hormone is lost in the bicarbonate washes.

Grollman and Firor (9) have described a method which they recommend because of its simplicity and large yield of hormone obtained. It is essentially the same as that described by Kutz

(6). Following the bicarbonate wash of the benzene-soluble fraction as in the Kutz procedure, Grollman and Firor wash with N HCl before suspending the benzene-soluble fraction in water. They emphasize the necessity of obtaining glands immediately after slaughter and of working at a very low temperature to prevent enzymatic destruction. We were able to obtain 750 dog units per kilo of gland by their method (Experiment 20). This is about 50 per cent of the yield which can be obtained with our method from autolyzed glands and less than 50 per cent of the yield which can be obtained from fresh tissue. The low yield obtained with the method of Grollman and Firor is due to the losses in the discarded fractions. We recovered 600 dog units per kilo with our method from the tissue residue and aqueous residue after extracting with acetone (including refluxing) and benzene respectively. This does not take into consideration the loss encountered in the bicarbonate washes.

Kendall and his coworkers (7, 8) extracted with acetone made 0.2 N with H_2SO_4. The object of the acid was to liberate the cortical hormone from an assumed complex, for they state (7): "The hormone exists only in small part in a freely extractable form; most of it is firmly bound to the protein fraction and is only liberated in the presence of free acid." The yield of hormone obtained with their technique was 1100 dog units per kilo. The yield was less than that which was obtained with neutral alcohol or acetone (compare Experiments 10, 16, and 19). Tissue which had been extracted twice with neutral alcohol failed to yield a significant amount of activity when further extracted either by the method of Kendall *et al.* (Experiment 17) or by our method (see diagram). These results demonstrate that all of the cortical hormone is freely extractable with neutral alcohol or acetone. Kendall *et al.* (7) state further that the benzene-soluble fraction contains only "insignificant traces of the hormone." Aqueous residues from our standard method (see diagram) were fractionated by Kendall's procedure. Only an additional 75 dog units were obtained (Experiment 18), showing clearly that all the activity was in the benzene-soluble fraction.

Separation of Adrenalin from Cortical Hormone—In most of the methods now in use for the preparation of the cortical hormone the bulk of the adrenalin is separated by selective distribution between immiscible solvents. Kendall *et al.* (7, 8) remove most of the adrenalin by basic lead precipitation. The really significant fractionation step, however, is the separation of the cortical hormone from the last traces of adrenalin which carry through the fractionation procedures. We have employed permutit for this purpose. As has already been mentioned, Kutz (6) removed adrenalin by washing a benzene solution of the active fraction with sodium bicarbonate solution, while Grollman and Firor (9) wash with both sodium bicarbonate and hydrochloric acid. A serious objection to such a separation is the loss of cortical hormone in the washes as evidenced by the relatively low yield. In distribution studies we have found the coefficient between benzene and aqueous bicarbonate or acid to be 1:3 or 1:4. Kendall *et al.* (7, 8) removed the last traces of adrenalin by grinding an acetone solution of the active fraction with lead nitrate and potassium carbonate. The remaining fraction still contains impurities which are readily removed with permutit. Hartman and Brownell (15) stated that much of the physiological potency was lost when permutit was used to separate adrenalin and the cortical hormone. No data have as yet been presented to substantiate this claim. If permutit is used properly, the loss of cortical hormone cannot be detected by available methods of determination. Permutit enjoys the distinct advantage in that it readily removes many impurities other than adrenalin from the active fraction. The relative efficiency of the several methods discussed is brought out in the data recorded under Experiments 4, 15, 16, and 20. In earlier work (3) we suggested the separation of adrenalin from the cortical hormone by washing an ether solution of the active fraction with 0.1 N NaOH. This fractionation step was abandoned when results with the present method of assay showed that most of the hormone was destroyed by this concentration of alkali.

Recovery of Adrenalin—Adrenalin can be recovered from the aqueous residue following benzene extraction. It has been our practise to concentrate the first and second alcohol extracts separately, since only the first extract contains sufficient adrenalin to make the recovery worth while. The yield of adrenalin based on the weight of the fresh glands is 0.12 to 0.17 per cent.

On the Nature of Impurities Present in Adrenal Extract—In

1931 Houssay and Marenzi (18) pointed out that choline was absent from adrenal extracts prepared by our method. We had never made direct tests for choline, since it is readily removed from alcoholic solution by permutit (19). Eagle (20), apparently unacquainted with the work of Houssay and Marenzi (18), concluded that this type of adrenal extract contained considerable quantities of choline. He based this conclusion upon results obtained with "2 microscopic tests, a precipitation test, and a color test" but neglected to specify the exact nature of the tests. It is a well recognized fact that choline has no specific chemical reaction by which it can be identified (21, 22). The two most characteristic reactions of choline are (1) splitting off of trimethylamine on alkaline hydrolysis, and (2) formation of the characteristic choline periodide crystals (23). Our extract gives a negative trimethylamine test. It gives several of the non-specific reactions which are also given by choline; *i.e.*, precipitation with Staněk's reagent, a positive alloxan reaction, precipitation in alcohol with mercuric chloride or platinic chloride, and an atypical positive test with Kraut's reagent. The first two reactions can be obtained equally well with a solution containing the mild oxidation products of adrenalin (0.1 per cent). The precipitate obtained with mercuric chloride or platinic chloride does not give the Rosenheim periodide test, which can be obtained readily with the corresponding salts of choline. To prove the presence of choline in adrenal extract it is necessary to isolate it in the form of one of its salts and to obtain analytical data for its identification. This we have attempted to do.

25 cc. of adrenal extract[4] representing 1 kilo of fresh beef adrenal glands were concentrated to semidryness on a water bath following the addition of 0.5 cc. of 0.1 N HCl. The residue was brought to constant weight (63.7 mg.) in a vacuum over sulfuric acid and extracted thoroughly with a total of 10 cc. of absolute ethyl alcohol. To 8.5 cc. of the filtered alcohol extract (representing 850 gm. of whole beef adrenal) 400 mg. of platinum chloride dissolved in 1 cc. of absolute ethyl alcohol were added. Precipitation was complete in 30 minutes. The light tan, amorphous

---

[4] The extract used in this experiment had no sodium chloride or preservative added. This sample was prepared from aliquots of five individual preparations of 4 kilos each.

precipitate was collected at the centrifuge, washed three times with 2 cc. portions of absolute ethyl alcohol, and dried in a vacuum over sulfuric acid. It weighed 11.6 mg. It gave a negative Rosenheim periodide reaction for choline. Analysis[5] of the chloroplatinate gave the following results.

$(C_5H_{14}NOCl)_2 \cdot PtCl_4$.	Calculated.	N 4.55,	Pt 31.67
	Found.	" 8.74,	" 29.33

These analytical data show clearly that the chloroplatinate is not choline chloroplatinate. The negative Rosenheim periodide reaction shows that the precipitate does not consist of a mixture of choline chloroplatinate and other chloroplatinates which might conceivably account for the analytical figures. In control experiments, the contamination of the chloroplatinate with choline chloroplatinate could be detected by this reaction when as little as 0.002 per cent choline hydrochloride was added to the original adrenal extract. These data demonstrate that choline is not present in our extract in detectable amounts.[6] Further work on the nature of this nitrogenous fraction is in progress. The quantities of chloroplatinate obtained from a series of extracts varied from 3.0 to 13.7 mg. per kilo of gland. Chloroplatinates were obtained in a similar manner from extracts prepared by the methods of Grollman and Firor (9) and Kendall *et al.* (7, 8). Both of these products, however, gave a positive Rosenheim periodide reaction for choline.

Cleghorn (10) described histamine-like reactions with extracts which he prepared. He observed the typical depressor effect of histamine after the extract had been exposed for 1 hour to 1 N NaOH. We obtained a negative Pauly reaction with 8.4 mg. of the water-soluble fraction equivalent to 90 gm. of cortex, and a negative Knoop-Hunter reaction with 14.0 mg., equivalent to 150 gm. of cortex. In numerous adrenalin bioassays (blood pressure reaction in the anesthetized dog and cat) on our extracts no evidence was obtained for the presence of histamine or a histamine-like substance. The product from as much as 4 kilos of glands when administered in a single intravenous injection to a 10 kilo dog had no untoward effects.

---

[5] Microanalytical data were obtained through the courtesy of Dr. Oskar Wintersteiner.

[6] Three samples of eschatin (Parke, Davis and Company, lot Nos. 094896-A, 854234, and 295) were examined in a similar manner. In no case were we able to establish the presence of choline.

SUMMARY

A quantitative study has been made of the extraction of the cortical hormone of the adrenal gland. Assays were conducted on a standardized series of adrenalectomized dogs. The maximum yield of cortical hormone which can be obtained from beef adrenal gland by any available method is about 2500 dog units per kilo. The method giving this yield is approximately 75 per cent efficient. All of the hormone can be extracted from the gland with neutral alcohol or acetone. Methods of separating the two hormones of the gland and the nature of the impurities present in the extract are discussed.

BIBLIOGRAPHY

1. Pfiffner, J. J., and Swingle, W. W., *Anat. Rec.*, **44**, 225 (1929).
2. Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, **96**, 153, 164, 180 (1931).
3. Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, **98**, 144 (1931).
4. Harrop, G. A., Pfiffner, J. J., Weinstein, A., and Swingle, W. W., *Proc. Soc. Exp. Biol. and Med.*, **89**, 710 (1932).
5. Pfiffner, J. J., Swingle, W. W., and Vars, H. M., *J. Biol. Chem.*, **104**, 701 (1934).
6. Kutz, R. L., *Proc. Soc. Exp. Biol. and Med.*, **29**, 91 (1931).
7. Kendall, E. C., and McKenzie, B. F., *Proc. Staff Meetings Mayo Clin.*, **8**, 90 (1933).
8. Kendall, E. C., Mason, H. L., McKenzie, B. F., and Myers, C. S., *J. Biol. Chem.*, **100**, lix (1933).
9. Grollman, A., and Firor, W. M., *J. Biol. Chem.*, **100**, 429 (1933).
10. Cleghorn, R. A., *J. Physiol.*, **75**, 413 (1932).
11. Hartman, F. A., and Brownell, K. A., *Proc. Soc. Exp. Biol. and Med.*, **27**, 938 (1930); *Am. J. Physiol.*, **95**, 670 (1930).
12. Zwemer, R. L., Agate, F. J., Jr., and Schroeder, H. A., *Proc. Soc. Exp. Biol. and Med.*, **28**, 721 (1931); *Endocrinology*, **15**, 382 (1931).
13. Hartman, F. A., MacArthur, C. G., and Hartman, W. E., *Proc. Soc. Exp. Biol. and Med.*, **25**, 69 (1927).
14. Stewart, G. N., and Rogoff, J. M., *Science*, **66**, 327 (1927).
15. Hartman, F. A., and Brownell, K. A., *Science*, **73**, 620 (1931).
16. Hartman, F. A., Thorn, G. W., Lockie, L. M., Greene, C. W., and Bowen, B. D., *J. Am. Med. Assn.*, **98**, 788 (1932).
17. Rogoff, J. M., *J. Am. Med. Assn.*, **99**, 1309 (1932).

18. Houssay, B. A., and Marenzi, A. D., *Compt. rend. Soc. biol.*, **107**, 1199 (1931).
19. Whitehorn, J. C., *J. Biol. Chem.*, **56**, 751 (1923).
20. Eagle, E., *Proc. Soc. Exp. Biol. and Med.*, **30**, 1094 (1933).
21. Hoppe-Seyler, F., and Thierfelder, H., Handbuch der physiologisch- und pathologisch-chemischen Analyse für Aertze und Studierende, Berlin, 9th edition, 196 (1924).
22. Alles, G. A., *Physiol. Rev.*, **14**, 276 (1934).
23. Rosenheim, O., *J. Physiol.*, **33**, 220 (1905–06); **35**, 465 (1906–07).

EXTRACTION STUDIES ON THE ADRENAL CORTICAL HORMONE

II. YIELD FROM GLANDS OF VARIOUS SPECIES*

By HARRY M. VARS, A. R. TAYLOR, and J. J. PFIFFNER

(From the Biological Laboratory, Princeton University, Princeton)

(Received for publication, June 13, 1934)

In earlier work (1, 2) the maximum yield of hormone obtained from fresh whole beef adrenal glands was shown to be about 2500 dog units per kilo. In recovery experiments it was demonstrated that the extraction method was 75 per cent efficient; *i.e.*, 75 per cent of previously assayed hormone added to adrenal tissue devoid of hormone activity could be recovered in the form of an extract suitable for assay purposes. It seemed desirable to determine the yield of hormone from the adrenal glands of other species. The present report is a summary of the results obtained with the glands of man, horse, ox, sheep, hog, dog, and shark.

Material

The adrenals from man were supplied to us by Dr. R. S. Ferguson of the Memorial Hospital, New York. The adrenal tissue was collected at 107 necropsies. Death was due to a variety of causes. No glands from cases of cancer or pregnancy were included. The glands were frozen after dissecting off the excess fat. The collection extended over a period of 80 days. The glands were received January 23, 1933.

The horse glands were collected at the abattoir of Chappel Brothers, Inc., Rockford, Illinois, through the courtesy of Dr. A. E. Meyer and were supplied to us April 29, 1933, by Dr. S. J.

---

* This investigation was supported by a grant from the Josiah Macy, Jr., Foundation.

We wish to express our appreciation to the management and research staff of Parke, Davis and Company for their generous cooperation.

Martin of the Medical School, University of Wisconsin. After trimming off the excess fat the glands were chilled thoroughly before shipping.

The ox, sheep, and hog adrenals were collected from animals at time of slaughtering. These glands were frozen after trimming off the excess fat and shipped to the laboratory packed in dry ice. The ox glands were received on March 16, 1932, and the sheep and hog glands on March 2, 1933.

The dog glands were collected from the carcasses of animals sacrificed at the Trenton dog pound. The dogs were killed by the municipal authorities with carbon monoxide. The glands were removed within 1 hour after death. After chilling and removing extraneous tissue, the glands were frozen at −10°. The collection extended over a period of 66 days, the period ending July 17, 1933.

TABLE I

Yield of Cortical Hormone from Adrenal Glands of Various Animals

	Estimated average body weight	Average weight of adrenal tissue		Weight of glands extracted	Average yield			
		Per animal	Per kilo body weight		Per kilo gland	Per mg. active fraction	Per pair of adrenal glands	Per kilo body weight
	kg.	*gm.*	*gm.*	*gm.*	*dog units*	*dog units*	*dog units*	*dog unit*
Man	70	14.0	0.20	1500	<165		<2.3	<0.03
Horse	500	42.2	0.08	2500	1500	25	73.8	0.15
Ox	420	20.0	0.05	4000	2500	42	50.0	0.12
Sheep	60	2.7	0.05	2345	3500	70	9.6	0.16
Hog	110	5.4	0.05	2250	3500	59	18.9	0.17
Dog	9	1.2	0.13	395.7	2500	5	3.2	0.33
Shark	13.5	2.2*	0.16	163.6	<500		<1.1*	<0.07

* Interrenal body.

The interrenal tissue of the shark (*Galeus vulgaris*) was collected at the Staatliche Biologische Anstalt auf Helgoland under the direction of Dr. A. Hinrichs during the late summer of 1933. The animals (75) were brought to the laboratory alive and the interrenal tissue removed at the time of killing. The tissue was finely chopped and shipped in 2.5 volumes of ethyl alcohol.

Methods of Extraction and Assay

The tissues were extracted by the usual method (3) with certain minor modifications discussed more recently (2). Since the quantities of available tissue varied appreciably, 163.6 to 4000 gm., the yield of hormone obtained from varying quantities of ox adrenal glands was determined as a check on extraction technique. It was found that the yield of hormone from 500 gm. of beef adrenal glands with the technique employed was essentially the same as that obtained from a 4000 gm. lot. The dog method of assay was employed (4). Each assay was checked on at least two animals. All test animals were standardized against a single preparation of hormone, that of the ox, recorded in Table I.

DISCUSSION

The figures recorded in Table I under the heading "Average yield" represent the average of the "holding" and "failing" dosage levels employed in the assay; *i.e.*, if in the course of the assay the test animals were maintained adequately on a dosage level corresponding to 3000 dog units per kilo of tissue but lapsed into insufficiency on reduction of the dosage level to that corresponding to 4000 dog units, the "average yield" was accepted as 3500 dog units per kilo. The yield of hormone obtained from the glands of horse, ox, sheep, hog, and dog are of the same order of magnitude (1500 to 3500 dog units per kilo of gland). The tissues of man and the shark contained negligible amounts in comparison with the glands of other species, <7 and <20 per cent that of the ox. With the available material only a maximum limit could be established for the hormone content of the glands in these two species. The amount of hormone present may have been zero. In the case of man there are several possibilities to explain the low yield. We are inclined to feel that the glands were depleted of their hormone content at the time of death. One could explain the low yield by assuming destruction of the hormone by autolytic processes if it were not for the fact that a good yield of hormone can be obtained from ox glands after 48 hours autolysis at room temperature (about 23°) (1). The yield from a clear cell hypernephroma (weight 537 gm.), supplied to us by Dr. R. S. Ferguson, was no greater than that obtained from other human autopsy material. Studies on the hormone content of adrenal tumors should be made with tissues removed at operation rather than at necropsy.

The only obvious time for the destruction of the hormone in the interrenal tissue was while en route (about 2 weeks). Control experiments demonstrate that beef adrenal glands can be ground and stored in 2.5 volumes of ethyl alcohol for 2 weeks at room temperature without a detectable loss in potency. Cleghorn (5) was unable to demonstrate the presence of the hormone in extracts which he prepared from the interrenal body of skate (*Raja clavata*). He used the survival of the adrenalectomized cat as the physiological criterion of potency and demonstrated the adequacy of his extraction technique by the preparation of physiologically potent extracts from ox adrenals. Grollman, Firor, and Grollman (6) criticized Cleghorn's work on the grounds (1) that the hormone was probably destroyed before the tissue was dissected from the fish and (2) that the extraction technique was inadequate. These workers prepared extracts from the interrenal tissue of the skate (*Raja stabuliforis*, *Raja diaphanes*, and *Raja erinacea*), using a method of their own which is a modification of one described by Kutz (7). They presented data on six adrenalectomized rats (three injected and three control). The three experimental rats injected daily for a period of 7 days with relatively large doses gained an average of about 7 gm. and died 10, 11, and 12 days following operation. The three control rats survived 5, 6, and 8 days and showed no gain in weight. These data were interpreted as conclusive proof of the presence of the cortical hormone in interrenal tissue. From the extirpation experiments of Biedl (8) and Kisch (9) it is extremely probable that the cortical hormone is present in the interrenal body. We do not feel that its presence has been established adequately by any extraction and substitution data presented to date.

Most workers consider the interrenal body to be adrenalin-free. The material with which we worked gave several of the usual colorimetric tests for adrenalin.

The estimated average body weights accepted in Table I for the ox, sheep, and hog are those recorded for animals at slaughtering (10); that for the horse was made at the abattoir (11); the average body weight of the shark was determined at killing; that of the dog is the average of body weight recorded by Rogoff and Stewart ((12) Tables I and II). The average weight of adrenal tissue found by these authors in a series of 67 dogs was 1.21 gm. In the present work the average weight of the adrenal tissue was 1.23 gm. in a series of 322 dogs. It seems justifiable therefore to accept the average body weight of 9 kilos for purposes of calculation, although it is realized that even in the same species there is no strict correlation between body and adrenal weight.

In comparing the species on the basis of the amount of adrenal tissue per unit of body weight it will be seen that the horse, ox, sheep, and hog fall into one group while man, dog, and shark fall into another. The relatively smaller amount of adrenal tissue per unit of body weight in the first group may be due to the fact that the animals in the group, with the probable exception of the horse, are for the most part fattened for slaughter.

Another interesting comparison of the various species can be made on the basis of the yield of hormone per kilo of body weight. The values for the horse, ox, sheep, and hog are reasonably constant, 0.12 to 0.17 dog unit per kilo of body weight. The values for man and the shark are very low, <0.03 to <0.07 dog unit, whereas the value for the dog is 0.33.

The data on the dog are of particular interest, for they give an indication of the rate of synthesis of the hormone in the adrenal cortex. The dog unit is the minimum daily kilo dose required for maintenance under standard conditions. Since the dog gland at any one time contains only one-third of this amount, the period of depletion in the absence of continued synthesis would be about 8 hours. If we assume the hormone requirement of the other species to be comparable to that of the dog, then the depletion periods calculated with the data available would be as follows: man <43 minutes, horse 3.6 hours, ox 2.9 hours, sheep 3.8 hours, hog 4.1 hours, dog 8 hours, and shark <1.7 hours.

SUMMARY

Extracts were prepared from the adrenal glands of man, horse, ox, sheep, hog, and dog, and from the interrenal body of the shark. The extracts were assayed for their cortical hormone content on a standardized series of adrenalectomized dogs. The rate of synthesis of the cortical hormone in the adrenal gland is discussed.

BIBLIOGRAPHY

1. Pfiffner, J. J., Vars, H. M., Bott, P. A., and Swingle, W. W., *Proc. Soc. Exp. Biol. and Med.*, **29**, 998 (1932).
2. Pfiffner, J. J., Vars, H. M., and Taylor, A. R., *J. Biol. Chem.*, **106**, 625 (1934).
3. Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, **96**, 153, 164, 180 (1931); **98**, 144 (1931).
4. Pfiffner, J. J., Swingle, W. W., and Vars, H. M., *J. Biol. Chem.*, **104**, 701 (1934).
5. Cleghorn, R. A., *J. Physiol.*, **75**, 413 (1932).
6. Grollman, A., Firor, W. M., and Grollman, E., *Am. J. Physiol.*, **108**, 237 (1934).
7. Kutz, R. L., *Proc. Soc. Exp. Biol. and Med.*, **29**, 91 (1931).
8. Biedl, A., Innere Sekretion, 2nd edition, Berlin and Vienna (1913).
9. Kisch, B., *Endokrinologie*, **1**, 31 (1928).
10. United States Department of Agriculture yearbook 1932, Washington, 782, 805, 795 (1933).
11. Meyer, A. E., private communication.
12. Rogoff, J. M., and Stewart, G. N., *Am. J. Physiol.*, **78**, 683 (1926).

EXTRACTION STUDIES ON THE ADRENAL CORTICAL HORMONE

III. DISTRIBUTION STUDIES*

By J. J. PFIFFNER and HARRY M. VARS

(*From the Biological Laboratory, Princeton University, Princeton*)

(Received for publication, June 13, 1934)

In earlier communications (1, 2, 3) methods of extraction for beef adrenal glands were described which yielded physiologically active extracts assaying from 30 to 40 dog units of cortical hormone per mg. of active fraction. These figures for activity are based on the solid content of the aqueous extracts. The potency of the permutit-purified fractions before separating the water-soluble from the water-insoluble fraction was much less, usually assaying between 10 and 20 dog units per mg. From the standpoint of preparing the hormone in a form suitable for physiological or clinical study this separation is quite adequate but it is an undesirable and unwieldy fractionation step in isolation work, since it leaves the hormone dissolved in a relatively large volume of water. Methods, therefore, were investigated which would be applicable directly to the permutit-purified fraction. Some degree of purification with little loss of potency could be effected by precipitating inert material from alcoholic solution with petroleum ether or other hydrocarbon solvent; *e.g.*, 470 mg. of an active fraction assaying 8000 dog units (17 dog units per mg.) were precipitated from 10 cc. of absolute ethyl alcohol with 90 cc. of hexane. A brown, sticky precipitate was thrown down which was

---

* This investigation was supported by a grant from the Josiah Macy, Jr., Foundation.

We wish to express our appreciation to the management and research staff of Parke, Davis and Company for their generous cooperation.

A preliminary report of some of these data appeared previously (Pfiffner, J. J., Vars, H. M., Bott, P. A., and Swingle, W. W., *Proc. Soc. Exp. Biol. and Med.*, **29**, 1267 (1932)).

reprecipitated from 5 cc. of absolute ethyl alcohol with 45 cc. of hexane. The soluble fraction weighed 370 mg. and contained all of the activity (22 dog units per mg.). This fraction could in turn be divided into a water-soluble and water-insoluble fraction. The water-soluble fraction weighed 150 mg. and contained 8000 dog units (53 dog units per mg.). Products of this degree of potency, however, could be obtained by simply fractionating the permutit-purified material directly with water. It seemed desirable to study selective distribution methods before attempting further purification with organic solvents.

Methods

The system of distribution used in the study summarized in Table I was as follows: The active fraction was taken up in 100 cc. of ether and 25 cc. of an aqueous solution (neutral, acid, or alkaline). The aqueous phase was drawn off and the ether solution washed five times with fresh 25 cc. portions. Each wash consisted of a vigorous rotation or agitation for a period of 5 minutes. The aqueous washes were washed in succession with a 100 cc. portion of ether. The ether solution and ether wash were combined and constitute the "First ether-soluble fraction" of Table I. The combined aqueous washes constitute the "Water-soluble fraction" of Table I. In each instance an aliquot of the water-soluble fraction was neutralized and used in collecting the assay data indicated. The pH of the remainder of the fraction was adjusted as indicated in Table I and washed six times with ether, the volume of each wash being two-thirds the volume of the aqueous phase. These combined ether washes constitute the "Second ether-soluble fraction" of Table I. The ether-soluble fractions were prepared for assay by distilling off the ether under reduced pressure, dissolving the fraction in alcohol, adding the necessary quantity of water, removing the alcohol under reduced pressure, passing through a Seitz filter, and adding 0.8 per cent sodium chloride and 0.1 per cent benzoic acid. The second ether-soluble fraction was almost entirely water-soluble. In preparing the water-soluble fraction for assay, the neutralized aliquot was freed of ether by distillation under reduced pressure; the volume was then adjusted, and 0.8 per cent sodium chloride and 0.1 per cent benzoic acid added.

Table I

Purification of Cortical Hormone by Distribution between Ether and an Alkaline, Acid, or Neutral Aqueous Phase

The starting material in each experiment consisted of 0.47 gm. of permutit-purified fraction (representing 4 kilos of beef adrenal glands) containing 8000 dog units (17 dog units per mg.).

Experiment No.	First ether-soluble fraction				Water-soluble fraction					Reaction of aqueous phase	Second ether-soluble fraction			
	Weight	Physiological activity			Added solute	Weight	Physiological activity				Weight	Physiological activity		
		Total	Per mg.	Recovery			Total	Per mg.	Recovery			Total	Per mg.	Recovery
	mg.	*dog units*	*dog units*	*per cent*	*gm. per l.*	*mg.*	*dog units*	*dog units*	*per cent*	*pH*	*mg.*	*dog units*	*dog units*	*per cent*
1	200	<500	<2.5	<6	2 (NaOH)	270	750	2.8	9	12.7*				
2	350	1000	2.9	13	20 ($NaHCO_3$)	120	6000	50	75	8.2*	40	8,000	200	100
3	350	1500	4.3	19	1.8 (HCl)	120	7000	58	88	6.7†	30	>6,000	>200	>75
										1.3*	30	8,000	270	100
4	310					160				6.6	40	10,000	250	125

* Approximate only.

† Aliquot of water-soluble fraction adjusted to pH 6.7 before ether wash.

The physiological potency was determined by the dog method of assay (4). All test animals were standardized against a single preparation of hormone.

DISCUSSION

The results are recorded in Table I. When 0.05 N NaOH was used as the aqueous phase, less than 15 per cent of the activity could be recovered. The hormone was exposed to the alkali a maximum period of 1 hour. It is obvious from these findings that such a procedure as this could not be profitably used in separating adrenalin from the cortical hormone. The earlier conclusion (2) that the two hormones could be separated in such a manner was based on the fact that large doses of the ether-soluble fraction relieved the symptoms of adrenal insufficiency in the cat. Even though less than 6 per cent of the activity could be accounted for in the ether-soluble fraction, this would be quite ample to relieve insufficiency in the dosages employed. The water-soluble fraction was not tested at the time because of the adrenalin present.

Experiment 2 demonstrated the stability of the hormone in the presence of 2 per cent $NaHCO_3$. Most of the activity was found in the water-soluble fraction, from which it could be readily washed back into ether at an alkaline pH. Apparently the hormone is non-acidic. In Experiment 3 it is seen that the activity could be washed readily from ether into 0.05 N HCl. It could then be washed back into ether, either with or without neutralization of the acid, indicating the hormone to be non-basic in character. Experiment 4 showed that the hormone could be washed from an ether solution into water and back into ether.

By means of such distribution procedures the physiological potency can be increased 10 to 15 times with a loss of less than 25 per cent. The weight of a dog unit was reduced from 60 to about 5 micrograms.

If one assumes a distribution coefficient of the hormone between ether and water of 1:3, the recoveries found in Table I agree fairly well with the calculated recoveries. For Experiments 2 and 3, the first ether-soluble fraction contained 13 and 19 per cent respectively, calculated 12 per cent; the water-soluble fraction contained 75 and 88 per cent, calculated 88 per cent; the second ether-soluble fraction contained 100, >75, 100, and 125 per cent, calculated 77 per cent.

The distribution coefficient of the hormone between ether and water was determined directly as follows: 102 mg. of permutit-purified material assaying 2400 dog units were allowed to distribute between 50 cc. of ether and 50 cc. of water. The system was shaken repeatedly during a period of 1 hour. The two phases were separated and each phase prepared for assay in the usual manner. The aqueous phase was found to contain 2000 dog units, while the other phase assayed 500 dog units, giving a water to ether ratio of 4:1. This experiment was repeated with water and benzene with essentially the same findings. A difference in the ratios 1:3 and 1:4 is within the experimental error of the assay method. The significant point is that the ratio definitely favors the aqueous phase in an ether- (or benzene-) water system.

From the foregoing data a method of fractionation was devised for relatively large quantities of tissue. The details are brought out in the following example. 7.45 gm. of the permutit-purified fraction obtained from 69 kilos of whole beef adrenal glands, and containing 85,000 ($>$69,000 $<$104,000 by assay) dog units, were transferred to a separatory funnel with 75 cc. of ether and 50 cc. of 0.05 N HCl. A small amount of tarry material which did not dissolve was discarded. The aqueous acid phase was drawn off and the ether solution washed seven times with 25 cc. portions of 0.05 N HCl. The remaining ether-soluble fraction weighed 4.7 gm. The combined acid washes (225 cc.) were neutralized to pH 6.0 and concentrated to a volume of 50 cc. under reduced pressure at a bath temperature of 45–50°. 2.5 cc. of N HCl were added to the concentrate and it was extracted ten times with 100 cc. portions of ether. The aqueous acid-soluble fraction weighed 1.7 gm. The physiological activity is found in the ether washes. The combined ether washes were brought to 50 cc. and the hormone transferred to bicarbonate solution by extracting eight times with 25 cc. portions of 2 per cent $NaHCO_3$. The inactive ether-soluble fraction weighed 0.25 gm. The combined bicarbonate washes (200 cc.) were brought to pH 5.3 with hydrochloric acid and concentrated to 50 cc. in the usual manner. 1 gm. of $NaHCO_3$ was added and the solution extracted ten times with 100 cc. portions of ether. The inactive aqueous bicarbonate-soluble fraction weighed 0.4 gm. The ether washes were combined and all solvent removed by distillation under reduced pressure at a low temperature. Water was removed at the end-stage of the distillation with the aid of absolute ethyl alcohol. The residue was dried in a vacuum over sulfuric acid. It weighed 670 mg. and contained 84,000 ($>$67,000 $<$101,000 by assay) dog units. In this particular instance the weight of the active fraction was reduced from 7450 mg. to 670 mg. with no significant loss of activity. The product when reduced to a powder is amorphous and light tan in color.

The permutit-purified fractions from lots of glands ranging from 16 to 100 kilos have been carried through a similar fractionation procedure. In working with smaller quantities of material the product is practically colorless. With larger quantities, however, the product retains a small amount of pigment. In some of the earlier preparations water was used in the fractionation without the addition of hydrochloric acid or sodium bicarbonate. Feathery or needle-shaped crystals were formed in these products on slow evaporation of ether solutions. However, this particular type of crystalline material was never observed in later products in the preparation of which acid and bicarbonate were used. Chemical properties of highly potent concentrates will be discussed in a later communication.

In purifying the permutit fraction from smaller quantities of gland (16 kilos) by distribution procedures the yield ranged from 4 to 8 mg. per kilo, depending upon the method used in preparing the permutit fraction. The products assayed 200 to 400 dog units per mg. When the permutit fraction from larger quantities (75 to 100 kilos) was purified by this procedure, the yield was 6 to 10 mg. per kilo and the products assayed 100 to 200 dog units per mg.

Comment

In these distribution experiments the hormone behaves as a non-polar compound. It is apparently neither an acid nor a base. The fact that the hormone exhibits a distribution coefficient between water and ether (or benzene) of a magnitude of approximately 3:1 or 4:1 explains in part the relatively low yields of hormone obtained by the fractionation method suggested by Kutz (5) and modified by Grollman and Firor (6). In both of these methods adrenalin is removed by washing a benzene solution of the active fraction with aqueous sodium bicarbonate or hydrochloric acid or with both. These workers discarded the aqueous washings. Kendall *et al.* (7) suggested the use of acid saturated with sodium chloride. In our experience the hormone can be readily removed from an ether solution by washing with 0.05 N HCl saturated with sodium chloride. Distribution procedures can be substituted for permutit fractionation in separating adrenalin from the cortical hormone. However, the fact that the distribution ratio of the cortical hormone is preponderantly in favor of the aqueous phase in a water-ether (or water-benzene) system, means that severe losses will be encountered unless the distribution technique is designed to recover the cortical hormone from the bicarbonate or acid washings. We have found it unprofitable to introduce a distribution procedure before permutit fractionation because of the mechanical difficulties due to emulsion formation and the amount of time involved.

SUMMARY

Methods of purifying the adrenal cortical hormone are described, consisting of selective distribution between a neutral, acid, or slightly alkaline aqueous phase and an immiscible solvent phase. Amorphous products free of adrenalin and containing 200 dog units of cortical hormone per mg. have been prepared.

Acknowledgment is due to Mr. A. R. Taylor for his assistance in the preparation of the adrenal extracts used in this study.

BIBLIOGRAPHY

1. Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, **96**, 153, 164, 180 (1931).
2. Swingle, W. W., and Pfiffner, J. J., *Am. J. Physiol.*, **98**, 144 (1931).
3. Pfiffner, J. J., Vars, H. M., Bott, P. A., and Swingle, W. W., *Proc. Soc. Exp. Biol. and Med.*, **29**, 998 (1932).
4. Pfiffner, J. J., Swingle, W. W., and Vars, H. M., *J. Biol. Chem.*, **104**, 701 (1934).
5. Kutz, R. L., *Proc. Soc. Exp. Biol. and Med.*, **29**, 91 (1931).
6. Grollman, A., and Firor, W. M., *J. Biol. Chem.*, **100**, 429 (1933).
7. Kendall, E. C., Mason, H. L., McKenzie, B. F., Myers, C. S., and Koelsche, G. A., *J. Biol. Chem.*, **105**, xlv (1934); *Proc. Staff Meetings Mayo Clin.*, **9**, 245 (1934).

STUDIES ON THE CARBOHYDRATE METABOLISM OF THE GOAT

THE BLOOD SUGAR AND THE INORGANIC PHOSPHATE*

By JESSIE T. CUTLER

(*From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville*)

(Received for publication, April 30, 1934)

Levels of sugar in the blood considerably lower than those found in man and the laboratory animals commonly studied have been repeatedly observed in goats in our laboratory. The normal resting blood sugar of non-fasted goats has, as a rule, been found to be below 50 mg. per 100 cc. Frequently the levels are lower than those at which other animals show symptoms of hypoglycemic shock. It seemed that such low sugar levels might be indicative of a further peculiarity in the manner in which the goat utilizes carbohydrate. Consequently, a study of a number of phases of the carbohydrate metabolism of these animals has been undertaken. The present paper includes the results of an investigation of the following points: (1) the difference in sugar content of arterial and venous blood; (2) the renal threshold for sugar; (3) the mobilization of sugar following excitement, or administration of adrenalin; (4) the hyperglycemia resulting from administration of glucose; (5) the effects of insulin and the resulting hypoglycemia; and (6) the changes in the level of the inorganic phosphates in the blood which result from the administration of glucose, adrenalin, or insulin.

Normal Blood Sugar—We have made 150 determinations of the normal resting blood sugar in twenty-six goats. The figures range from 24 to 65 mg. per 100 cc. The majority of the figures are between 40 and 50 mg. per 100 cc. It was found that if reasonable care was exercised to prevent exciting the goats before taking the samples, the levels were nearly always below the latter figure. The average of the figures is 46 mg. per 100 cc.

---

* The experimental data in this report are taken from a thesis submitted by the author in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Vanderbilt University, June, 1934.

Only two earlier references to the normal blood sugar level of the goat have been noted. Abderhalden (1) reports a concentration of sugar of 0.829 part per 1000, and Bang (2), one of 0.08 part per 100 in the blood of the goat. These figures are considerably higher than those which we are reporting, but were determined by methods which are not at the present day considered accurate.

The goats used in our studies were obtained from four different localities, and were of various breeds. Normal males, castrated males, and females, both lactating and non-lactating, were included. The low blood sugars were persistent whether the goats had been at pasture or had been kept for several months in the laboratory on a diet of oats and hay.

The sugar in the blood was determined routinely by Benedict's method (5). However, because the figures obtained were unusual, they were repeatedly checked by the use of several other well established methods. Folin's ferricyanide method (11) and the method of Shaffer and Hartmann (18) gave figures which checked very well with those obtained by Benedict's method. Moreover, approximately the same figures were obtained whether the determinations were carried out on protein-free filtrates prepared by the use of the Folin-Wu tungstic acid precipitation procedure (12) or the zinc sulfate protein precipitation of Somogyi (19). Typical figures obtained by these various methods are presented in Table I.

There is apparently no substance in goat blood which interferes with the determination of glucose, as is indicated by the fact that glucose added to the blood can be quantitatively recovered.

The factor of loss of sugar due to rapid glycolysis has been ruled out in several ways. In the first place, data to be presented later show that glycolysis occurs exceptionally slowly in goat blood. In the second place, glycolysis was prevented in most of the samples used for sugar determination, either by such rapid handling that not even an anticoagulant was needed, or by the addition of sodium fluoride to the blood. Parallel determinations on fluorized and non-fluorized portions of the same samples showed no significant differences in sugar content, even when the samples were allowed to stand at room temperature for an hour.

The figures presented above, therefore, apparently indicate the true level of reducing sugar in the blood of goats. They do not, however, preclude the possibility that additional sugar may be present in a non-reducing form, yet is available to the animal for metabolic purposes.

Arterial-Venous Difference in Sugar Content—The figures presented above were obtained on venous blood. In several cases, samples were taken as nearly simultaneously as possible from the saphenous vein and from the left ventricle of the heart. Comparative sugar determinations on these samples show sugar levels in the ventricular blood only 2 to 4 mg. per 100 cc. higher than those in venous blood. These differences are wholly in accord with those observed in other animals. Moreover, we found that insulin, which increases the utilization of sugar, does not cause the arterial venous difference of sugar content in the blood of the goats to increase beyond the normal limits. Therefore, the low sugar concentration in venous blood cannot be explained on the basis of an unusually rapid withdrawal of sugar from the blood by the tissues.

TABLE I

Blood Sugar of Normal Goats. Determinations by Several Methods

Sample	Benedict method	Folin method	Sample	Benedict method, Folin-Wu filtrate	Benedict method, Somogyi filtrate	Shaffer-Hartmann method
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
A	47	42	F	42	42	48
B	44	41	G	42	43	53
C	43	44	H	42	43	40
D	46	46	I	51	51	53
E	46	47	J	46	49	50

Renal Threshold for Sugar—An extremely low renal threshold is not the cause of the low blood sugar. This is indicated by the fact that numerous samples of urine taken from goats more or less at random did not contain sugar. Further evidence was obtained by experiments on four goats in which the renal threshold for sugar was determined. We were not able to catheterize goats and were forced to use anesthetized animals. Amytal was found to be unsatisfactory for maintaining anesthesia in goats, and we therefore resorted to barbital. This anesthetic had only a slight effect on the blood sugar, as evidenced by the fact that the levels after anesthetization and cannulation of a ureter were only slightly higher than those obtained before the procedures were instituted. Before the anesthetic was given, the blood sugar levels of the four goats were 45, 44, 35, and 46 mg. per 100 cc. After the operation they were 61, 46, 44, and 47 mg., respectively.

The renal threshold was determined in the following manner. Urine was obtained from the ureteral cannula and immediately tested for sugar by Benedict's method (4). A small dose of glucose, about 0.06 gm. per kilo, was then injected intravenously. A blood sample was taken immediately and the urine examined for sugar at 2 to 5 minute intervals for about 30 minutes. If the urine did not give a positive test for sugar, a slightly larger dose of glucose was given and the procedure repeated. After sugar appeared in the urine, tests were made as frequently as possible, and and as soon as sugar-free urine was again obtained, another blood sample was taken. Thus, we were able to determine the approximate levels of blood sugar at which sugar appeared in, and disappeared from, the urine.

In the four goats, sugar was noted in the urine at blood sugar levels of 130, 81, 112, and 115 mg. per 100 cc., and had disappeared at 80, 67, 89, and 87 mg., respectively. These levels indicate a threshold considerably lower than that reported for the dog, the rabbit, and man. The threshold in the goat is, however, almost as much higher than the normal blood sugar level as it is in other animals. Excretion of sugar in the urine, therefore, cannot be the direct cause of the low blood sugar.

Mobilization of Sugar—Having established the fact that the goat is able to maintain its normal activity with an unusually low concentration of sugar in the blood, we wished to know whether in an emergency it can draw upon its carbohydrate reserves in a normal manner. The administration of large doses of adrenalin to most animals is followed by an increase in blood sugar. Likewise, fright or excitement results in hyperglycemia. We have found that goats respond to the administration of adrenalin and to excitement with a hyperglycemia which is comparable to that seen in the dog. In Chart 1, typical curves are given which illustrate the rise in blood sugar that occurs in the goat after the subcutaneous injection of 0.1 mg. of adrenalin per kilo and after moderate excitement.

The figures obtained from these procedures indicate that though the goat maintains normal metabolism with a very low blood sugar, it is like other animals in being able to call into the blood stream reserves of sugar in an emergency.

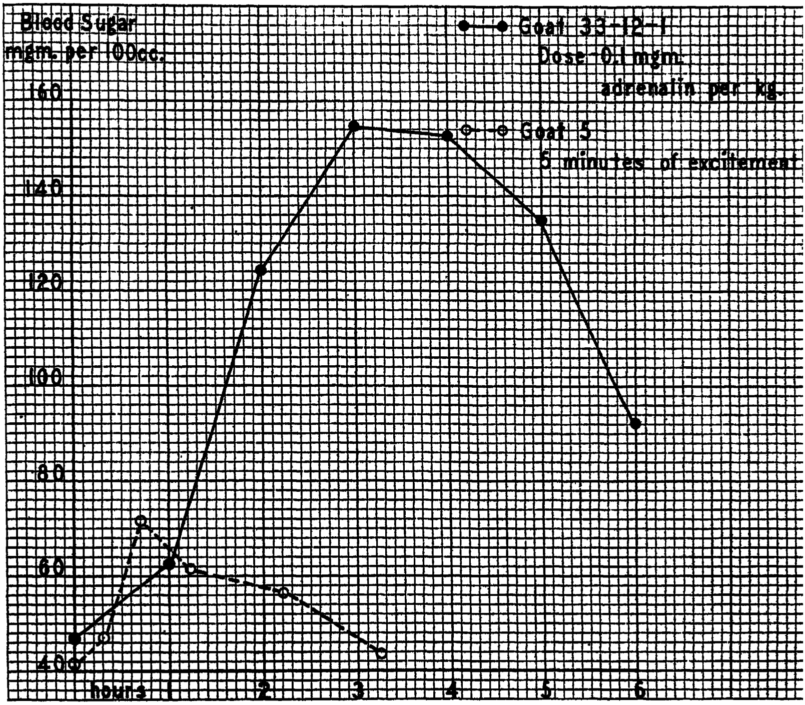


Chart 1. Effect of adrenalin and excitement on the blood sugar of goats

Administration of Sugar—To investigate the rate at which goats remove excess sugar from the blood stream, we administered glucose, either orally or by vein, and made subsequent determinations of the blood sugar concentration at short intervals. When sugar is given by mouth to goats, the resulting hyperglycemia is slight and rather late in appearing. One of the factors influencing the blood sugar level after the oral ingestion of glucose is the rate of absorption from the gastrointestinal tract. The goat is a ruminant and it is possible, therefore, that the rate of absorption is relatively slow. If so, this may explain why the blood sugar rise is so slight and so late in appearing.

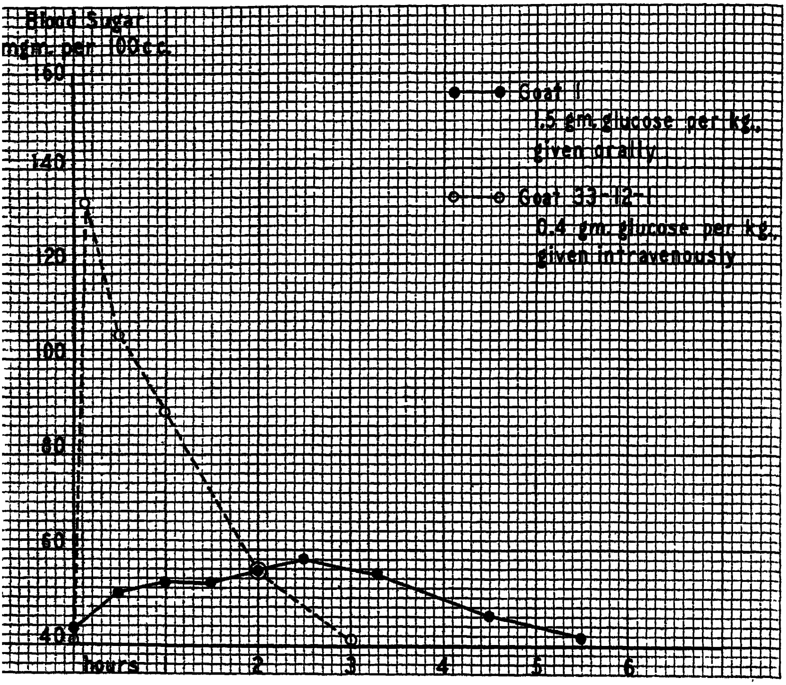


Chart 2. Effect of oral and intravenous administration of glucose on the blood sugar of goats.

When comparable amounts of glucose are administered by vein to the goat and the dog, the blood sugar response in the two animals is similar. The abrupt initial rise is followed by a return to the normal level in 1½ to 3 hours. In Chart 2 the blood sugar changes in the goat after oral and intravenous administration of glucose are illustrated. From the results of these experiments we may conclude that in the goat excess sugar can be removed from the blood at a normal rate.

Effects of Insulin—The reaction of the goat to insulin is unusual in two respects: (1) exceptionally large doses of insulin are required to produce shock; and (2) shock occurs only after a blood sugar level between 10 and 20 mg. per 100 cc. has been maintained for a period of 5 to 8 hours.

The smallest dose of insulin with which we have produced shock in the goat is 4 units per kilo. Even larger doses than this have frequently failed to produce symptoms. Scott, Ferrill, Rogoff, and Barnes (17), in a recent study on the sensitivity of dogs to insulin, reported that 2 units of insulin per kilo produced convulsions in dogs in nearly every case, and that smaller doses would suffice in certain dogs. Therefore, at least twice as much insulin per kilo of body weight is required to produce shock in the goat as in the dog.

When large doses of insulin are administered to goats, the blood sugar falls rapidly, within 1 or 2 hours, to a level between 10 and 20 mg. per 100 cc. At this stage, the animals appear perfectly normal in every respect, and continue to do so for some time. The blood sugar usually remains at approximately the same low level for several hours. If, within 4 or 5 hours, it begins to rise toward its normal level, no symptoms are ever noted in the animal. If, however, the dose of insulin is sufficient to keep the blood sugar concentration well below 20 mg. per 100 cc. for 5 to 8 hours, symptoms of shock will be noted. Although we have sometimes used extremely large doses of insulin, we have never been able to produce shock in the goat without this 5 to 8 hour period of hypoglycemia. In two cases, doses of 10 units per kilo and 12 units per kilo were administered, but symptoms appeared only 8 and 9 hours, respectively, after the insulin was given. We can, as yet, offer no explanation of why the goat is able to maintain his normal activity for several hours with a blood sugar so remarkably low, and then without further lowering, manifests shock.

The symptoms of shock in the goat are those of apathy and sluggishness, followed by coma. We have never seen convulsions, and only in a few cases, slight signs of restlessness in the early stages. The animals usually salivate profusely. The symptoms can be relieved promptly and completely by injection of glucose, and as far as can be observed, there are no after effects.

The hypoglycemia resulting in the goat from injection of insulin and its relationship to the onset of symptoms are illustrated in Chart 3.

The studies of the blood sugar have not, thus far, revealed the nature of the abnormality in the carbohydrate metabolism of the goat. The peculiarity of the reaction of this animal to insulin is, however, still further suggestive of the existence of such an abnormality. Therefore, another criterion for the comparison of the carbohydrate metabolism of the goat with that of other animals was sought.

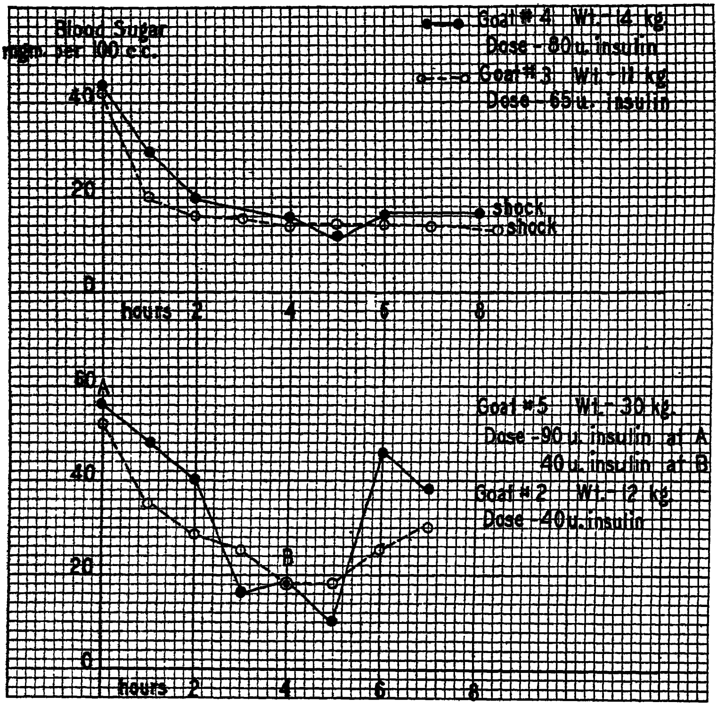


Chart 3. Blood sugar of goats after insulin administration. The blood sugar curves in the upper part of the chart resulted from the injection of doses of insulin large enough to produce shock; those in the lower part resulted from doses which did not produce symptoms.

Numerous reports in the past decade have indicated that phosphorus plays an important rôle in carbohydrate metabolism. One type of evidence that this is so is found in the fact that the changes in blood sugar which result from the administration of glucose, adrenalin, or insulin are accompanied by characteristic changes in the level of the inorganic phosphates of the blood. Therefore, we have studied the changes produced by these procedures in the level of the inorganic phosphorus in the blood of the goat.

The normal concentration of the inorganic phosphate in the blood of the goats which were used for these studies was considerably higher than that in the blood of the dogs used as control animals. Our figures for goat blood range from 3.2 to 11.1 mg. of phosphorus per 100 cc. with an average of 6.0 mg., while those for dog blood vary from 2.2 to 4.9 mg. per 100 cc. with an average of 3.2 mg. The determinations were made by Briggs' modification (8) of the Bell-Doisy method (3). We believe that the high levels in the goat blood result, at least in part, from the diet. The figures were always considerably higher when the animals had been kept at pasture for some time than when they had been receiving the diet of oats and hay furnished in the animal quarters. The soil in this region is rich in phosphates, so that it is probable that the animals kept at pasture received a comparatively large amount of these salts.

Effect of Administration of Glucose on Inorganic Phosphate—Harrop and Benedict (13) reported in 1924 that the hyperglycemia resulting from the oral administration of glucose was followed by a marked reduction of the inorganic phosphate of the blood. Bolliger and Hartman (7) showed that the same change occurs after intravenous injection of glucose in normal animals, but that no such change takes place in depancreatized subjects. Hartman and Foster (14), as well as McCullagh and Van Alstine (15), have studied the changes in the inorganic phosphate of the blood after the administration of glucose to large numbers of patients. Both groups of investigators found that certain abnormalities of carbohydrate metabolism are reflected in an abnormal curve of the inorganic phosphate.

In our experiments, glucose was given intravenously to both goats and dogs in doses of 0.4 gm. per kilo. The intravenous route was chosen in order to avoid variations in the rate of intestinal absorption. Blood samples for the determination of sugar and inorganic phosphate were taken 10, 30, 60, 120, and 180 minutes after the injection.

In the blood of both species, the abrupt initial rise in blood sugar which results from the injection of glucose is followed by a marked reduction of the inorganic phosphate. The fall continues for 40 minutes to 2 hours, following which there is a return toward the normal level. The rise in phosphate does not usually begin until the blood sugar has fallen to a level only slightly in excess of the normal. The phosphate level is, as a rule, still somewhat depressed as long as 3 hours after the administration of glucose. In Chart 4, typical curves are presented of the inorganic phosphate

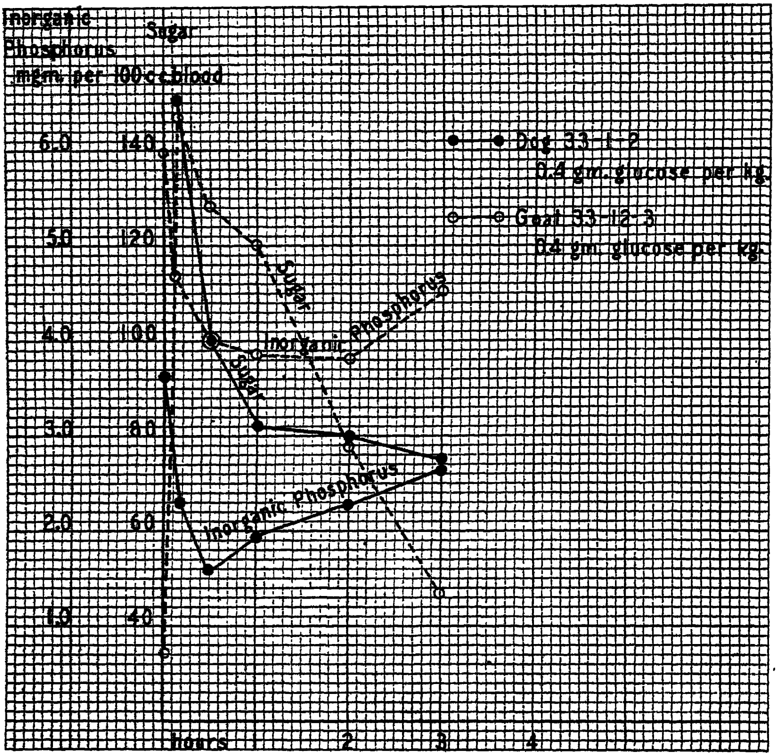


Chart 4. Effect of intravenous injection of glucose on the inorganic phosphate of the blood of the goat and the dog.

of the blood of the dog and the goat after the intravenous injection of 0.4 gm. of glucose per kilo. The changes which occur in the goat blood do not differ from those in dog blood and are similar to those reported in the literature.

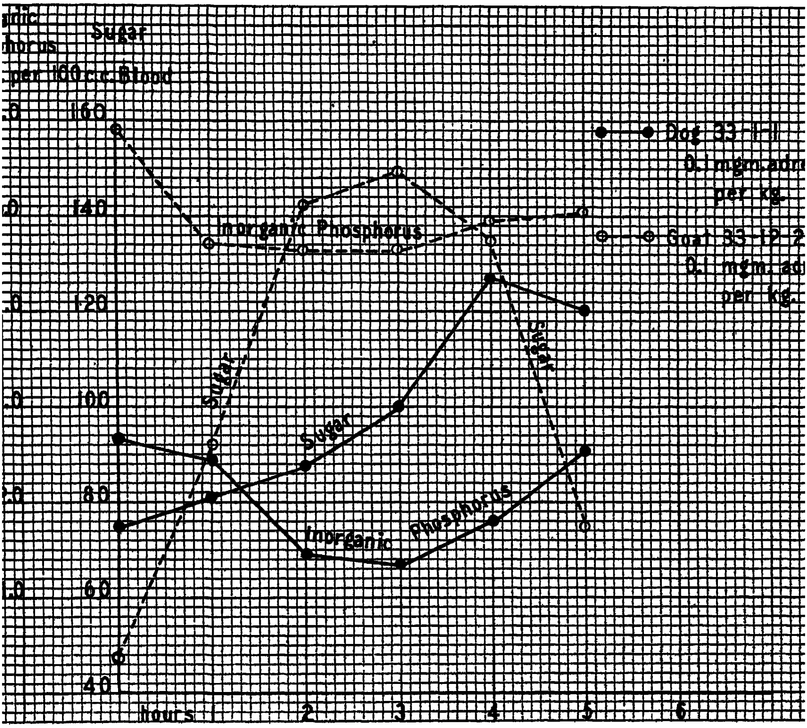


Chart 5. Effect of adrenalin on the inorganic phosphate in the blood of the dog and the goat.

Effect of Injection of Adrenalin—Perlzweig, Latham, and Keefer (16), Vollmer (21), and Bolliger and Hartman (7) reported that the injection of adrenalin results in a marked fall in the inorganic phosphate of the blood. Yamada (23) was unable to confirm this observation.

In Chart 5 are presented typical curves illustrating the changes in sugar and inorganic phosphate of the blood which result from the subcutaneous injection of adrenalin in dogs and goats. Adrenalin was given in doses of 0.1 mg. per kilo. Blood samples were taken hourly, following the injection, for 6 to 8 hours. The resulting curves in the two species are similar. Following the injection of adrenalin the blood sugar rises rapidly at first and then more slowly for 3 or 4 hours. During the same period the inorganic phosphate falls markedly. Both constituents then begin to return toward their normal levels.

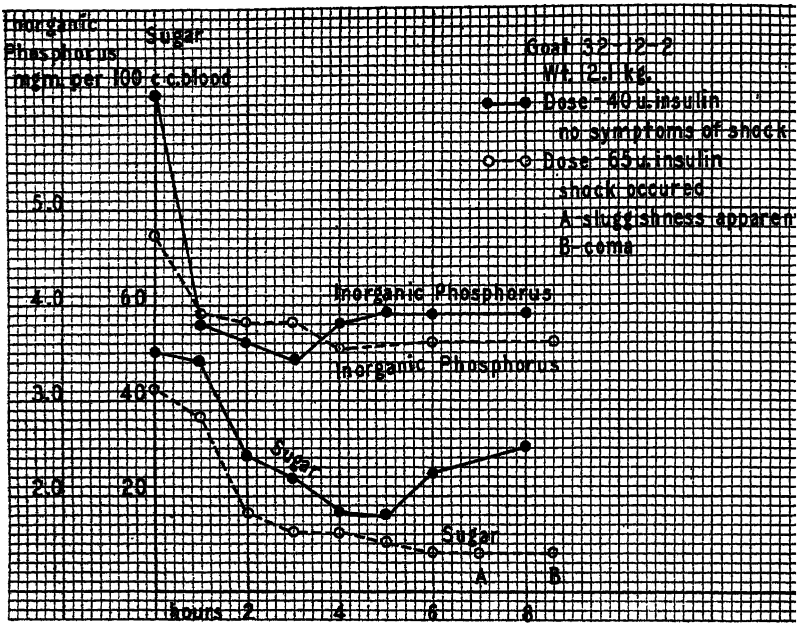


CHART 6. Effect of insulin on the inorganic phosphate in the blood of the goat.

Effect of Injection of Insulin—A marked fall in the inorganic phosphate of the blood following the administration of insulin was reported by Wigglesworth, Woodrow, Smith, and Winter (22). This observation has since been confirmed by many investigators (6, 9, 10, 13, 20, 23). Wigglesworth *et al.* found that the low level of the inorganic phosphate persists in the blood for many hours after the hypoglycemic convulsions have been relieved by glucose. Yamada (23) reported, however, that during unrelieved insulin convulsions the inorganic phosphate rises again to approach the normal level.

The large dose of insulin required and the greater severity and duration of hypoglycemia necessary to produce shock in goats make difficult any exact analysis of the comparative effect of insulin in goats and dogs. However, the marked fall in the inorganic phosphate of the blood which has been observed in other animals after the injection of insulin takes place also in the goat. The initial fall of both sugar and inorganic phosphate in goat blood is about equally great whether or not the dose of insulin is large enough to produce shock. When shock does not occur, the inorganic phosphate begins to rise at about the same time that the sugar starts to return to a more normal level. When shock occurs, the inorganic phosphate remains low until symptoms have set in and have been relieved by administration of glucose (Chart 6).

The data presented indicate that the inorganic phosphate of goat blood is altered in response to the administration of glucose, adrenalin, and insulin in the same manner as that observed in other animals. Not enough is yet known regarding the significance of the changes in inorganic phosphate in relation to the metabolism of carbohydrate to make it possible to say that these observations indicate a normal type of carbohydrate metabolism in the goat. They do not, however, furnish any evidence of metabolic abnormalities.

SUMMARY

The normal resting blood sugar of goats has been found to be between 24 and 65 mg. per 100 cc. The low blood sugar is not to be explained by an unusually efficient utilization of sugar by the tissues, nor by a remarkably low renal threshold. The blood sugar changes following excitement, the administration of adrenalin, or the administration of sugar are similar to those observed in the dog. Unusually large doses of insulin are required to produce shock in goats, and shock is manifested only when blood sugar levels between 10 and 20 mg. per 100 cc. have been maintained for 5 to 8 hours. The inorganic phosphorus of goat blood is

markedly lowered by the administration of glucose, adrenalin, or insulin.

BIBLIOGRAPHY

1. Abderhalden, E., Physiological chemistry, translated by Hall, W. T., and Defren, G., New York (1908).
2. Bang, I., Der Blutzucker, Wiesbaden (1913).
3. Bell, R. D., and Doisy, E. A., *J. Biol. Chem.*, **44**, 55 (1920).
4. Benedict, S. R., *J. Biol. Chem.*, **5**, 485 (1908-09).
5. Benedict, S. R., *J. Biol. Chem.*, **68**, 759 (1926).
6. Blatherwick, N. R., Bell, M., and Hill, E., *J. Biol. Chem.*, **61**, 241 (1924).
7. Bolliger, A., and Hartman, F. W., *J. Biol. Chem.*, **64**, 91 (1925).
8. Briggs, A. P., *J. Biol. Chem.*, **53**, 13 (1922).
9. Briggs, A. P., Koechig, I., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, **58**, 721 (1923-24).
10. Ellsworth, R., and Weinstein, A., *Bull. Johns Hopkins Hosp.*, **53**, 21 (1933).
11. Folin, O., *J. Biol. Chem.*, **81**, 231 (1929).
12. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
13. Harrop, G. A., Jr., and Benedict, E. M., *J. Biol. Chem.*, **59**, 683 (1924).
14. Hartman, F. W., and Foster, D. P., *Am. J. Clin. Path.*, **2**, 289 (1932).
15. McCullagh, R., and Van Alstine, L., *Am. J. Clin. Path.*, **2**, 277 (1932).
16. Perlzweig, W., Latham, E., and Keefer, C., *Proc. Soc. Exp. Biol. and Med.*, **21**, 33 (1923).
17. Scott, V. B., Ferrill, H. W., Rogoff, J. M., and Barnes, B. O., The sensitivity to insulin, presented before the American Physiological Society at New York (1934).
18. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, **45**, 365 (1920-21).
19. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
20. Staub, H., Günther, F., and Frölich, R., *Klin. Woch.*, **2**, 2337 (1923).
21. Vollmer, H., *Biochem. Z.*, **140**, 410 (1923).
22. Wigglesworth, V. B., Woodrow, C. E., Smith, W., and Winter, L. B., *J. Physiol.*, **57**, 447 (1923).
23. Yamada, S., *J. Biochem.*, Japan, **17**, 21 (1933).

CHEMICAL REACTIVITY OF CYSTINE AND ITS DERIVATIVES*

BY J. S. FRUTON† AND H. T. CLARKE

(*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, New York*)

(Received for publication, June 21, 1934)

In the studies here reported, investigation of the effect of substituent groups on the reactivity of the cystine molecule has been extended to include that of acid radicals attached to the amino group in both cystine and cysteine.

Sulfur Lability

The velocity of alkaline decomposition of cystine derivatives has been measured under the conditions adopted in previous studies (1). The results are given in Figs. 1 to 3. In the substituted cystine series (Fig. 1) the lability of the sulfur is increased by acyl substituents in the following order: carbobenzoxy < trichloroacetyl < (glycyl, alanyl, phenylhydantoic acid) < (α-bromopropionyl, chloroacetyl) < (acetyl, formyl). The induction period necessary for the accumulation of pyruvic acid is absent. The effect of acid substituents in general is to accelerate the formation of the aminoacrylic acid derivative formed as a primary decomposition product (2). As a substituent is already present on the amino group, pyruvic acid, which results from the aminoacrylic acid derivative, should have no effect on the rate of decomposition. This is shown to be the case by Curve I of Fig. 1. The presence of α-methyl hydroxylamine which, like arylhydrazines, restrains the velocity of the decomposition of cystine to its initial low value, likewise has no appreciable effect on the course of the reaction.

---

* This work was aided by a grant from the Chemical Foundation, Inc. The data in this paper are taken from a thesis submitted by J. S. Fruton in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

† W. J. Gies Fellow in Biological Chemistry, 1932-33.

A ring structure situated near the sulfur atom has a pronounced influence on the stability of the molecule. In the cases of cystine phenylhydantoin and the dianhydride of dialanylcystine, the great reactivity of which has been observed by Bergmann (2) and Brand and Sandberg (3), the end of the reaction is reached in less than 30 minutes. Evidently the ring strongly favors the formation of the aminoacrylic acid derivative. Owing to the possibility that this increased reactivity was due merely to the effect of a substituent on the carboxyl as well as on the amino group, the lability of the sulfur in glutathione was determined as well. This is intermediate between that for diacetylcystine and the ring compounds (Fig. 2). Nicolet (4) has found a behavior similar to that of the above two ring compounds in the case of cystine thiohydantoins. Andrews and Andrews (5) have studied the decomposition of cystine phenylhydantoin in pyridine

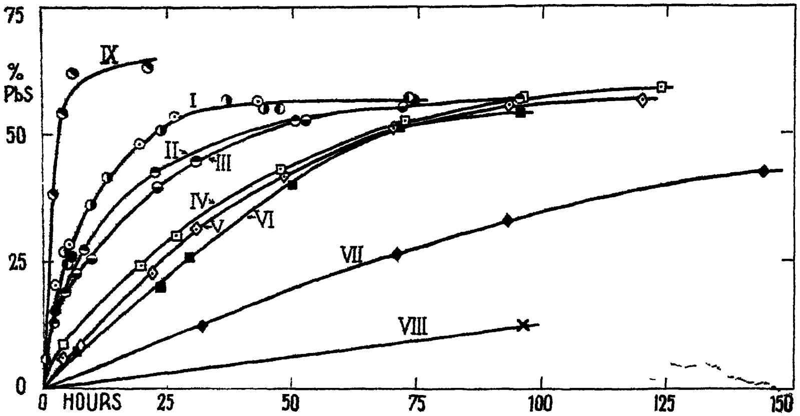


FIG. 1. Labile sulfur from cystine derivatives. Curve I, ◑ diformylcystine, ◐ diformylcystine + 2 moles of α-methylhydroxylamine, ⊙ diformylcystine + 5.75 moles of pyruvate, ● diacetylcystine; Curve II, dichloroacetylcystine; Curve III, di-α-bromopropionylcystine; Curve IV, cystine phenylhydantoic acid; Curve V, dialanylcystine; Curve VI, diglycylcystine; Curve VII, ditrichloroacetylcystine; Curve VIII, dicarbobenzoxycystine; Curve IX, dibenzylidenecystine.

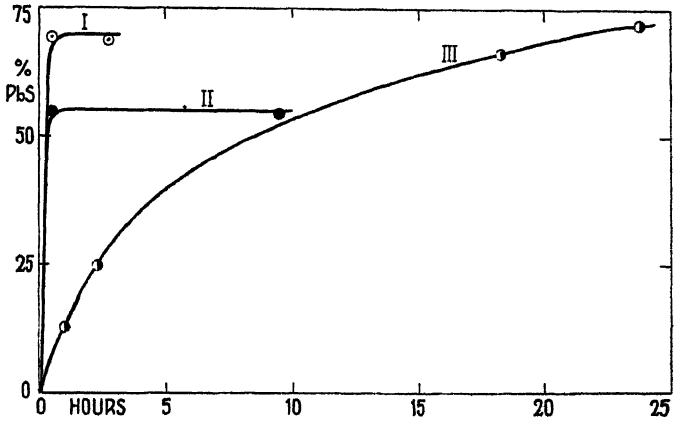


FIG. 2. Labile sulfur from cystine derivatives. Curve I, dialanylcystine anhydride; Curve II, cystine phenylhydantoin; Curve III, oxidized glutathione.

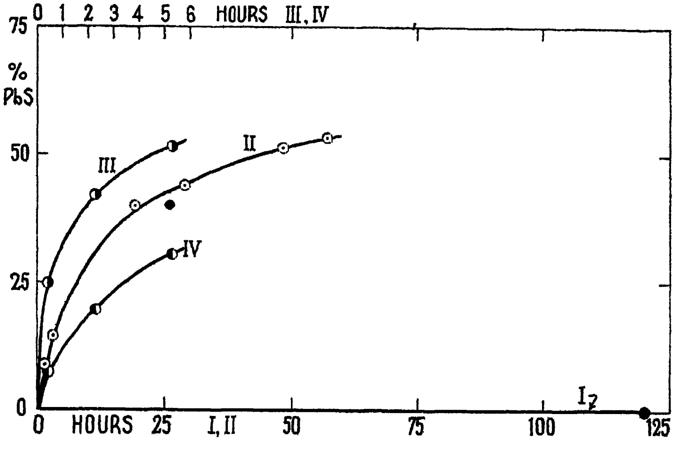


FIG. 3. Labile sulfur from benzenesulfonyl derivatives of cystine. Curve I, dibenzenesulfonylcystine (25°); Curve II, ⊙ di-N-methyldibenzenesulfonylcystine, ● di-N-benzyldibenzenesulfonylcystine (25°); Curve III, dibenzenesulfonylcystine (100°); Curve IV, benzenesulfonylcysteine (100°).

and showed the formation of pyruvic acid from the intermediate methylene compound in accord with the scheme outlined above. The remarkable stability of benzenesulfonylcystine (Fig. 3) to alkali may be attributed to the strongly polar nature of the benzenesulfonyl group which confers upon the residual hydrogen of the substituted amino group an acidic character so that it forms the sodium salt. When salt formation is blocked by replacement of the hydrogen by an alkyl group, the lability of the sulfur is greatly increased, and its rate of elimination becomes comparable to that of acetylcystine. While the increased lability in the case of the N-methylbenzenesulfonylcystine might be attributable to the increased basicity of the nitrogen atom, this cannot explain the similar reactivity of the corresponding N-benzyl derivative, for benzylamine has a dissociation constant no greater than that of ammonia.

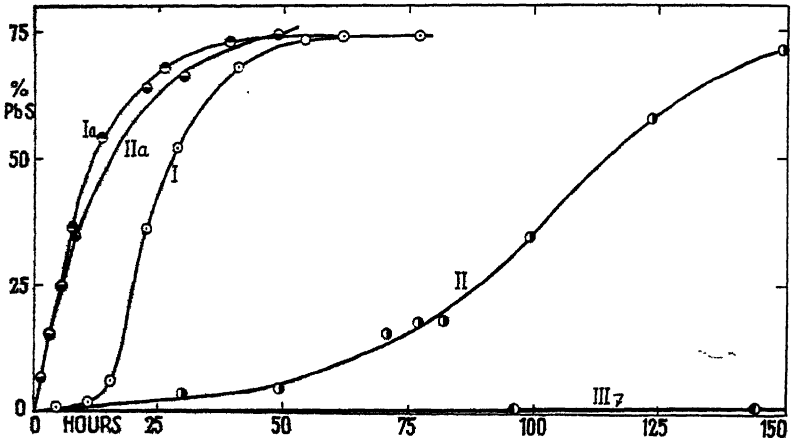


FIG. 4. Labile sulfur from cystine and cysteine. Curve I, cystine; Curve I, *a*, cystine + 5.75 moles of pyruvate; Curve II, cysteine; Curve II, *a*, cysteine + 11.5 moles of pyruvate; Curve III, cystine + 2 moles of α-methylhydroxylamine.

The rapid rate of decomposition of the benzylidene derivative is of interest.

The results with cysteine alone and with pyruvic acid (Curves II and II, *a*, Fig. 4) show that the induction period for the reduced form is much longer than that for the oxidized form, and the slope of the curve after the accelerating effect of pyruvic acid has set in is less steep.

In the experiments on the derivatives of cysteine essentially the same relationships were found as in the oxidized series (Fig. 5). The yields of PbS tended to approach the theoretical value. Here again the order was glutathione > (acetyl, formyl) > chloroacetyl > benzenesulfonyl. Similar differences were to be expected between the rates of decomposition for the reduced and oxidized forms. In the case of the compounds which decomposed quite rapidly at 25° only a slightly lower rate was observed for the reduced compounds. However, the reduced and the oxidized forms of the benzenesulfonyl derivatives (Fig. 3) showed a marked difference when their rates of decomposition were measured at 100°.

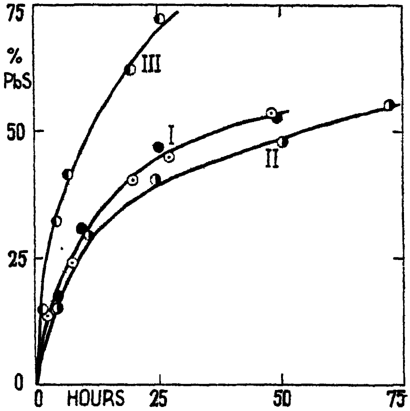


Fig. 5. Labile sulfur from cysteine derivatives. Curve I, ● acetylcysteine, ⊙ formylcysteine; Curve II, chloroacetylcysteine; Curve III, reduced glutathione.

An attempt was made to note the effect of the absence of either the amino or carboxyl group of cystine on the lability of the sulfur. With dithiodihydracrylic acid and diaminodiethyldisulfide (Fig. 6) the rates of decomposition were very slow. Homocystine, a sample of which was kindly transmitted to us by Dr. du Vigneaud, also decomposed at a very slow rate without the appearance of an induction period.

For cystine, glutathione, and dialanylcystine anhydride the maximum yields of PbS corresponded to approximately 70 per cent of the total sulfur, while in the runs on the other cystine derivatives in which an end-point was attained the yields were 55 to 60 per cent. In view of Schöberl's recent findings (6), it seems possible that the reaction is affected by the presence of oxygen.

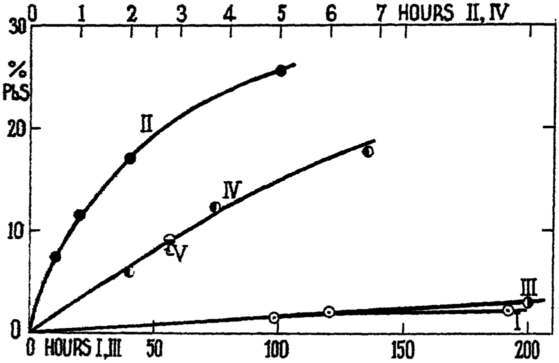


Fig. 6. Labile sulfur from various disulfides. Curve I, diaminodiethyldisulfide (25°); Curve II, diaminodiethyldisulfide (100°); Curve III, homocystine (25°); Curve IV, homocystine (100°); Curve V, dithiodihydracrylic acid (25°).

Autoxidation of Cysteine and Its Derivatives

It seemed of interest to learn whether the relationship between substitution and chemical reactivity observed in the studies on sulfur lability would be reflected in the rates of autoxidation of cysteine and its N-substituted derivatives. In these experiments the Warburg apparatus was employed.

The work of Mathews and Walker (7), Thunberg (8), Warburg and Sakuma (9), Sakuma (10), and Gerwe (11) showed that the reaction could be written $4R-SH + O_2 \rightarrow 2R-S-S-R + 2H_2O$, and that the reaction was extremely sensitive to metal catalysis. These findings are fully borne out in the experiments here reported.

In order to evaluate any comparative data on the autoxidation of cysteine and its derivatives it was necessary to carry out the experiments under as strictly analogous conditions as possible. Owing to the practical difficulty of totally excluding heavy metals, the determinations were made in the presence of a large constant concentration (0.0001 M) of Fe^{+++}, added as $FeCl_3$. Cysteine preparations from several sources after one recrystallization gave identical autoxidation rates on addition of this amount of iron.

Phosphate, borate, and veronal buffers were employed in M/15 solution. The pH readings, made immediately after the end of each autoxidation run, were obtained by means of the glass electrode and are reliable to 0.03 of a pH unit. It had been observed previously that no change in pH occurred during the autoxidation in these buffered solutions. The temperature in all cases was 25°; the volume of oxygen was corrected to 0° and 760 mm.

The figures for the oxygen uptake of cysteine (Table I) and thioglycolic acid (Table II) accord with those for first order reactions; those for formylcysteine and α-bromopropionylcysteine (Table III) conform with those for reactions of the second order. No explanation for these results is offered; with glutathione and benzenesulfonylcysteine no constants for either order were observed. If the rate of reaction were controlled by the concentration of ferrous-thiol complexes (12, 13), the oxygen uptake of cysteine and thioglycolic acid should conform to a zero order reaction.

The effect of the substituent on the amino group in the N-substituted cysteines upon the autoxidizability of the sulfhydryl group is qualitatively the same as on the lability to alkali. Formyl and similar groups, which labilize the thiol group, make its autoxidation more rapid; the benzenesulfonyl group, which stabilizes the sulfur to alkali, also decreases the rate of oxygen uptake.

In the studies on the effect of pH on the oxygen uptake of cysteine it was noted that the buffering agent used has a definite influence on the rate although it does not change its character. Thus phosphate buffers and borate buffers seem to check fairly well with each other, but give much slower rates for a given pH than the veronal buffer. It is possible that the phosphate and borate have a greater power of reducing the effective iron concentration than veronal; since knowledge on iron-veronal complexes is lacking, no decision can be made on this point.

The rate constants for a given buffer in the cysteine experiments show that for veronal the rate of autoxidation is practically independent of pH over the range pH 7 to 8; with borate or phosphate there is a similar "buffering" range with the center at

TABLE I

Autoxidation of Cysteine

Buffer	Time	pH 6.30		pH 7.0		pH 7.64		pH 8.06		pH 8.33		pH 8.79	
		O_2	K_1*	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1
	min.	*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*	
Veronal	20	23.0	1.96	78.5	5.80	86.0	6.47	88.2	6.50	106.2	8.34	124.4	10.06
	30	43.6	2.00	118.8	6.24	113.7	6.02	116.0	6.18	162.8	9.54	178.0	10.80
	40	64.8	2.28	144.8	6.12	145.4	6.19	151.2	6.46	197.3	9.52	231.0	10.21
	50	75.6	2.21	168.1	6.01	171.2	6.02	180.1	6.72	231.6	9.71	240.4	10.72
	60	92.1	2.33	191.1	6.07	191.8	6.15	197.8	6.40	246.1	9.46	266.5	11.39
	75	110.9	2.37	225.2	6.20	230.0	6.31	241.5	7.28	275.2	9.88	289.6	11.29
	90	128.3	2.30	243.3	6.16	244.0	6.17	258.7	7.04	292.5	9.98	336.0	
		pH 6.92		pH 7.28		pH 7.53		pH 7.67					
		O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1				
		c.mm.		*c.mm.*		*c.mm.*		*c.mm.*					
Phosphate	30	25.7	1.17	28.1	1.31	49.1	2.30	55.6	2.59				
	45	36.4	1.15	41.5	1.24	78.3	2.55	84.0	2.77				
	55	49.7	1.21	56.2	1.41	89.6	2.33	108.5	3.00				

		pH 7.50		pH 7.77		pH 7.87		pH 8.70		pH 9.10		pH 9.27		pH 9.64	
		O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1
		c.mm.		*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*	
Borate	20	26.5	1.76	43.6	3.01	53.1	3.66	42.9	3.15	58.0	4.13	53.6	3.88	41.6	2.90
	30	41.0	1.86	62.4	2.89			67.4	3.20	81.7	4.05	70.6	3.51	74.5	3.74
	40	51.6	1.83	82.8	3.12	87.1	3.25	88.6	3.25						
	50	61.7	1.74	91.5	2.80	99.7	3.01	104.5	3.23						
	60	69.6	1.68	106.0	2.78	106.3	2.78	117.6	3.06	148.3	4.19	147.3	4.19	136.8	3.76
	75	79.5	1.60	137.7	3.10	141.8	3.17	153.1	3.33	180.2	4.43	180.1	4.43	159.2	3.72
	90	98.1	1.66	159.8	3.10	176.2	3.51	174.6	3.50						
	105	118.9	1.80	190.0	3.16	197.8	3.15	211.1	4.11						

Concentration of cysteine, 0.0116 M. Theory requires 335 c.mm. of O_2; found, 336 c.mm.

* $K_1 = (K \text{ (monomolecular)}/2.303) \times 10^3$.

TABLE II

Autoxidation of Thioglycolic Acid

Time	pH 7.40		pH 8.03		pH 8.57		pH 9.16	
	O_2	K_1	O_2	K_1	O_2	K_1	O_2	K_1
min.	*c.mm.*		*c.mm.*		*c.mm.*		*c.mm.*	
20	19.7	1.18	38.3	2.49	65.5	4.43	90.2	6.11
30	33.7	1.35	61.6	2.64	98.2	4.69	135.6	6.45
40	41.0	1.37	73.7	2.52	131.5	4.85	156.0	6.31
60	60.5	1.29	103.8	2.51	174.0	4.72	200.9	6.18
80	81.9	1.41	125.5	2.39	210.4	4.63	243.1	6.19
100	94.0	1.32	161.3	2.57	248.5	4.91	272.0	6.37

Concentration, 0.0130 M; theory requires 355 c.mm. of O_2.

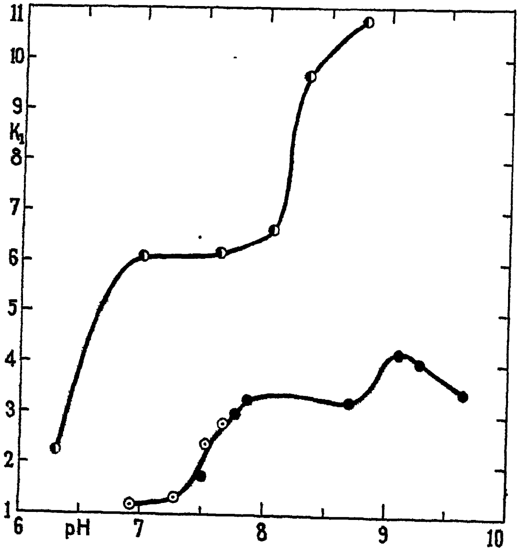


FIG. 7. pH dependence of cysteine autoxidation rate constants. ◐ veronal buffers, ⊙ phosphate buffers, ● borate buffers.

about pH 8.3 (Fig. 7). These findings contrast markedly with the results of Dixon and Tunnicliffe (14), who found a maximum rate at pH 7.4, but the discrepancy may be explainable by the presence of constant amounts of iron in the experiments here reported. It will be noted that in the case of the borate buffer

TABLE III

Autoxidation of Cysteine Derivatives

Formylcysteine, 0.0100 M; theory requires 280 c.mm. O_2; found, 278 c.mm. O_2							Bromopropionylcysteine, 0.0100 M; theory requires 280 c.mm. O_2; found, 283 c.mm. O_2					Glutathione, 0.0108 M; theory requires 300 c.mm. O_2; found, 299.0 c.mm. O_2			Benzenesulfonylcysteine	
Time	pH 7.31		pH 7.75		pH 8.31		Time	pH 7.35		pH 8.30		Time	pH 7.65	pH 8.62	pH	O_2 taken up in 125 min.
	O_2	K_2	O_2	K_2	O_2	K_2		O_2	K_2	O_2	K_2		O_2	O_2		
min.	*c.mm.*		*c.mm.*		*c.mm.*		*min.*	*c.mm.*		*c.mm.*		*min.*	*c.mm.*	*c.mm.*		*c.mm.*
10			176.0	17.0	240.0	68.0	10			209.1	28.4	10		53.7	7.08	8.3
15	45.5	1.27					20			248.7	36.5	20	10.3	150.0	7.63	27.6
20			212.6	17.6	261.5		30	59.3	0.89	263.2		30	19.5	254.1	7.76	31 4
25	58.8	1.06					45	80.1	0.87			45	30.7	299.0	8.67	41.7
30			228.0	15.9			60	101.9	0.93	283.0		60	54.8		9.18	54.6
40	83.2	1.07					90	132.6	0.97			90	79.6		9.47	68.1
60	111.4	1.11	268.3		278.0		125	143.4	0.87							
85	143.2	1.22														
125	156.5	1.02														

the rate begins to fall off after pH 9. This is probably due to decrease of iron concentration by precipitation as the hydroxide. The significance of the range of constant autoxidation rate is not clear, but may be associated with the amino group of the cysteine molecule. The values for this range lie close to the pK_b of 8.09 determined by Cannan and Knight (15). No such break is observed with the substituted cysteines nor with thioglycolic acid.[1]

In an attempt to study the autoxidation of ethyl mercaptan (Table IV), the rate was found to be almost independent of pH above 7.5 and apparently independent of iron concentration over the range 0.01 M to 0.0001 M Fe^{+++}. However, the end of the autoxidation could not be attained within a reasonable time, and calculations of the rate constants were fruitless.

TABLE IV

Autoxidation of Ethyl Mercaptan

O_2 values measured in c.mm. are given at different pH values with borate buffers.

pH	7.5		8.4		9.2	
Fe^{+++} concentration, M	0.0001	0.01	0.0001	0.01	0.0001	0.01
min.						
35	58.9	102.3	64.0	76.0	83.5	75.5
85	89.4	118.0	100.2	99.0	91.0	105.7
345	174.1	188.5	180.3	181.0	222.2	154.8
715	226.0	214.0	225.7	209.5	255.3	194.1

Oxidation-Reduction Studies

The experiments reported in this section concern the behavior of cysteine and several cysteine derivatives when they are allowed to react with reversible oxidation-reduction indicators. The original intention of these experiments was to study the kinetics of the reduction of some of the dyes by the sulfhydryl compounds and to observe whether the relationships found for sulfur lability and autoxidation as to the effect of substituents held for the anaerobic oxidation of these compounds as well. During the course of the work it was found that with certain of the dyes reduction by the sulfur compound reached stationary values.

---

[1] The results with thioglycolic acid are in agreement with those of Dixon and Tunnicliffe (14).

The oxidation-reduction potentials of the thiol-disulfide systems have been the subject of extensive electrometric investigation by Dixon and Quastel (16), Michaelis (17), Ghosh and his co-workers (18), and Green (19). The difficulties encountered in the use of metallic electrodes have led to the view, widely held, that the sulfhydryl-disulfide system is irreversible. For this reason the method of equilibration with oxidation-reduction indicators, suggested in 1926 by Conant (20), was adopted.

On the assumption that the reaction $2R-SH + D_o \rightleftharpoons R-S-S-R + D_r$ is reversible and by making use of the approach discussed in a recent communication from this laboratory (21) it was possible to calculate a characteristic potential for the thiol-disulfide system. The equilibrium constant K is given by $([D_o]\,[R-SH]^2)/([D_r]\,[R-S-S-R])$.

The dyes used were recrystallized commercial samples, with the exception of methyl viologen which was synthesized according to the method of Michaelis (22). The other indicators were gallocyanine,[2] gallophenine,[2] brilliant alizarin blue (23), indigo di- and tetrasulfonate (24), phenosafranine (25), and rosinduline 2-G[2] (26). The position of the absorption maxima at pH 7.4 of the dyes in the concentrations used in the equilibrium studies are given below.

Indigo disulfonate	610	Brilliant alizarin blue	590
" tetrasulfonate	590	Methyl viologen	600
Gallocyanine	620	Rosinduline 2-G	495
Gallophenine	590	Phenosafranine	520

Between pH 7.4 and pH 9.2 there was no significant variation in the position of the absorption maxima.

It was of importance to learn whether metal catalysis played a significant rôle in the kinetics of the decolorization. Toda (27) had observed that in the reaction between cysteine and methylene blue the addition of iron salts accelerated, while cyanide retarded, the decolorization. The experiments on cysteine with gallocyanine and indigo tetrasulfonate, both being reactions which result in nearly 100 per cent reduction of the dye for the concentrations used, show differences between the kinetics for the iron-catalyzed and cyanide-inhibited reactions which are quite definite but far smaller than for the aerobic oxidation. As in the experiments of Barron and Hoffman (28) with biological systems, the more positive the potential of a dye, the more rapidly it is decolorized. Thus brilliant cresyl blue ($E'_0 = +0.04$) was completely reduced in less than 5 minutes, under conditions in which the above indicators ($E'_0 = -0.132$ to -0.260) required several hours.

---

[2] We are grateful to the Winthrop Chemical Company of New York for kindly providing these dyes.

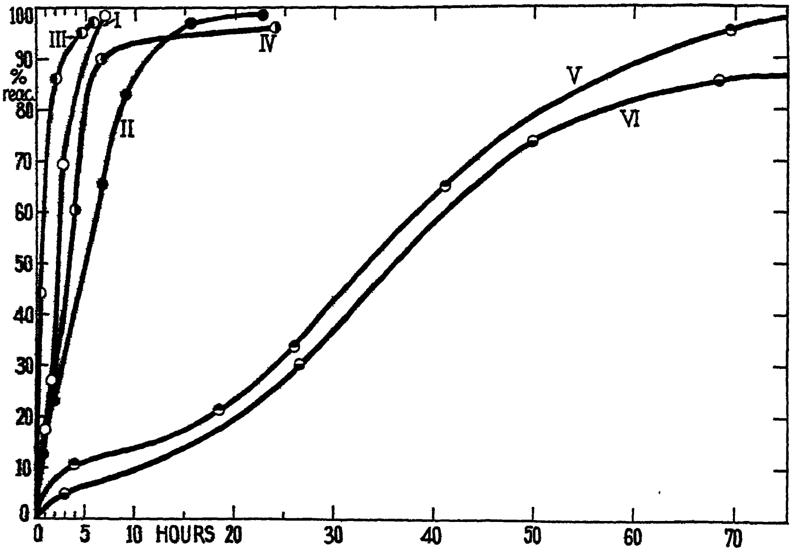


Fig. 8. Decolorization of indigo tetrasulfonate by —SH compounds. pH 7.15; molar concentration ratio 20:1; 0.00001 M Fe^{+++}. Curve I, cysteine; Curve II, cysteine + NaCN; Curve III, formylcysteine; Curve IV, glutathione; Curve V, thioglycolic acid; Curve VI, benzenesulfonylcysteine.

Experiments were carried out in order to compare the rates at which various —SH compounds reacted anaerobically with the oxidized dyes. Results with formylcysteine, thioglycolic acid, reduced glutathione, and benzenesulfonylcysteine with indigo tetrasulfonate are plotted in Fig. 8. The effect of substitution upon the velocity of these reactions is not unlike that in the autoxidation experiments.

One of the arguments for the irreversibility of the cystine-cysteine system was the fact that there was no oxidation-reduction indicator which cysteine would not reduce (17). The findings of Kendall and Nord (29), on the basis of which they claimed that indigo disulfonate was such a dye, were shown by Dixon and Tunnicliffe (30) to be due to admixture of oxygen in the nitrogen used. Our experiments are in agreement with Dixon's criticism, for it was found that indigo disulfonate is almost entirely reduced by cysteine at pH 7.15 if the ratio of concentrations of cysteine to dye is 20:1. However, dyes of much more negative potential, such as rosinduline or methyl viologen, are really incapable of appreciable reduction by means of cysteine in the above concentration ratio. Four of the dyes listed above, namely gallophenine,

TABLE V

Potentials of Cysteine

Indicator	pH	Time required	Equilibrium concentration of dye		$E'_0(d)$	$E'_0(s)$
			Oxidized	Reduced		
		hrs.	*per cent*	*per cent*	*volt*	*volt*
Indigo disulfonate................	7.15	106.5	4.0	96.0	−0.132	−0.227
Gallophenine......................		190.5	11.8	88.2	−0.151	−0.229
Brilliant alizarin blue............		118.25	35.4	64.6	−0.182	−0.236
Phenosafranine....................		74.5	95.0	5.0	−0.260	−0.234
Gallophenine......................	7.55	121	25.1	74.9	−0.175	−0.239
Brilliant alizarin blue............		79	54.7	45.3	−0.206	−0.245
Gallophenine......................	8.10	74.5	31.5	68.5	−0.208	−0.266
Brilliant alizarin blue............		64.5	47.3	52.7	−0.230	−0.275
Gallophenine......................	8.60	57.75	34.8	65.2	−0.238	−0.293
Brilliant alizarin blue............		45.5	64.6	35.4	−0.256	−0.285
Gallophenine......................	9.20	41	49.0	51.0	−0.273	−0.320
Brilliant alizarin blue............		31	70.0	30.0	−0.290	−0.315

Concentration of cysteine, 0.001 M; concentration of dye, 0.00005 M; temperature, 25°; phosphate and borate buffers employed.

brilliant alizarin blue, phenosafranine, and indigo disulfonate, gave with cysteine equilibria which yielded essentially identical values for E'_0 (*s*) at equal pH levels (Table V).

TABLE VI

Oxidation-Reduction Potentials of Cysteine Derivatives

—SH compound	Indicator	Time required	Equilibrium concentration of dye		$E'_0(s)$
			Oxidized	Reduced	
		hrs.	*per cent*	*per cent*	*volt*
Thioglycolic acid	Gallophenine	260	12.0	88.0	−0.229
	Brilliant alizarin blue	175	31.0	69.0	−0.240
	Phenosafranine	72.5	94.5	5.5	−0.236
Glutathione	Gallophenine	190	9.2	90.8	−0.233
	Brilliant alizarin blue	179	35.0	65.0	−0.237
Formylcysteine	Gallophenine	166	10.5	89.5	−0.231
	Brilliant alizarin blue	167	37.0	63.0	−0.232
Benzenesulfonylcysteine	Gallophenine	245	7.6	92.4	−0.236

Concentration of —SH compound, 0.001 M; concentration of dye, 0.00005 M; pH, 7.15; temperature, 25°.

TABLE VII

Effect of Fe^{+++} on Rate of Attainment of Equilibrium

Fe^{+++} concentration	Time required to reach equilibrium	Equilibrium concentration of dye	
		Oxidized	Reduced
M	*hrs.*	*per cent*	*per cent*
None added	297	15	85
5×10^{-6}	226	12	88
1×10^{-5}	192	14.5	85.5

Cysteine, 0.001 M; gallophenine, 0.00005 M; pH, 7.15; temperature, 25°.

Analogous experiments with thioglycolic acid, glutathione, formylcysteine, and benzenesulfonylcysteine (Table VI) showed that while the time required for the establishment of definite equilibria varied greatly with individual sulfhydryl compounds, the characteristic potentials of all fell within close limits. It appears that the portion of the molecule attached to the $-SH$ group exerts but little effect on the oxidation-reduction potential. A similar conclusion was reached by Ghosh *et al.* (18), employing electrolytic methods.

The dependence of the equilibrium upon metallic catalysts was studied by introducing small amounts of ferric iron into the gallophenine-cysteine system. The rate of attainment of equilibrium was slightly increased, but the equilibrium value was essentially unchanged (Table VII).

To test the conclusion that the reaction $R-SH \rightleftharpoons R-S-S-R$ is truly reversible, the interaction of disulfides and leuco dyes was studied, with the technique reported for the case of ascorbic acid (21). It is evident from the data presented in Table VIII that the potentials obtained by approaching equilibrium in the reaction $2R-SH + D_o \rightleftharpoons R-S-S-R + D_r$ from either direction are the same. As was to be expected from its low potential, the reduced methyl viologen[3] was completely oxidized by the $-SS-$ compounds, total decolorization occurring with formylcystine,

TABLE VIII

Reaction of Disulfide Compounds with Leuco Indicators

—SS— compound	Indicator (leuco)	Time required	Equilibrium concentration of dye		$E'_0(s)$	$E'_0(s)$ from opposite direction
			Oxidized	Reduced		
		min.	*per cent*	*per cent*	*volt*	*volt*
Cystine	Indigo disulfonate	60	69.0	31.0	−0.220	−0.227
	Gallophenine	10	81.1	18.9	−0.227	−0.229
Dithiodiglycolic acid	Indigo disulfonate	45	72.6	27.4	−0.215	
	Gallophenine	10	81.0	19.0	−0.227	−0.229
Formylcystine	Indigo disulfonate	30	69.7	30.3	−0.219	
	Gallophenine	10	74.0	26.0	−0.235	−0.231

Concentration of —SS— compound, 0.00005 M; concentration of leuco dye, 0.00005 M; pH, 7.15; temperature, 25°.

---

[3] With methyl viologen at pH 7.4 complete reduction could not be obtained by the catalytic method; comparison with a solution of methyl viologen of equal concentration reduced with excess $Na_2S_2O_4$ showed that approximately only 75 per cent had been reduced by H_2.

dithiodiglycolic acid, and oxidized glutathione within 10 minutes of the start of the reaction.

The reversibility of the system was further tested by altering the concentration ratios of sulfhydryl compound and dye, by employing the reaction of cysteine with phenosafranine. The results are given in Table IX.

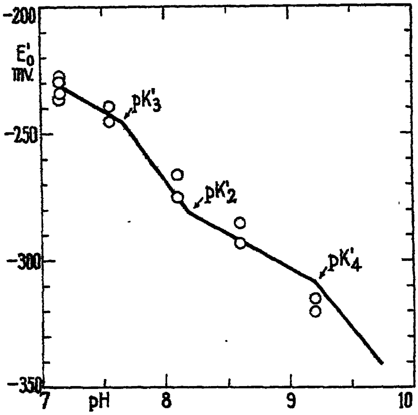


FIG. 9. pH dependence of oxidation-reduction potential of cysteine

TABLE IX

Effect of Concentration Change on Potential

Cysteine concentration	Equilibrium concentration of dye		$E'_0(s)$
	Oxidized	Reduced	
M	*per cent*	*per cent*	*volt*
1×10^{-3}	95	5	−0.234
3×10^{-3}	89.7	10.3	−0.224
4×10^{-3}	84.6	15.4	−0.228
5×10^{-3}	77.1	22.9	−0.234

Phenosafranine, 5×10^{-5} M; pH, 7.15; temperature, 25°.

The variation of the potential with pH in the case of cysteine is summarized in Fig. 9 from which it will be seen that the experimentally determined values agree satisfactorily with the curve calculated from the values for the dissociation constants of cysteine and cystine (15). According to Ghosh the reversibility of the

—SH—SS— system is established only on the alkaline side of pH 7 and is attained only when the —SH groups begin to dissociate. The complete experimental study of the pH dependence by the method employed in these experiments was prevented by the extreme slowness of the reaction between sulfhydryl groups and the oxidized dyes on the acid side of pH 7.

The potentials found for the —SH—SS— system by means of the oxidation-reduction indicators are much more positive than those obtained by Michaelis, Ghosh, or Green by the electrometric method. Since a definite interpretation of the processes occurring at a metallic electrode in the presence of the —SH—SS— system is still lacking, this discrepancy cannot be satisfactorily explained.

EXPERIMENTAL

The sulfur lability was determined by the method described by Clarke and Inouye (1). Unless otherwise stated, the temperature was 25°. The concentration of the disulfide compounds was 0.025 M and that of the sulfhydryl compounds 0.05 M.

Diformylcystine—12 gm. of cystine were mixed with 60 gm. of formic acid (95 to 99 per cent) and refluxed on the water bath for 3½ hours. Solution took about 1 hour with gradual browning. The reaction mixture was then placed in the ice chest, and after 9 days the crystalline precipitate which had formed was filtered off and recrystallized from hot water. M.p. 185–186° (uncorrected); yield 65 per cent of the theoretical. The yield was raised to 87 per cent by allowing the filtrate to stand at 0–5° for 2 weeks.

The same product was formed by the action of 30 cc. of acetic anhydride upon a solution of 6.0 gm. of cystine in 90 cc. of 90 per cent formic acid; after being allowed to stand for 2 hours at room temperature the mixture was diluted with water and evaporated to dryness under reduced pressure. The colorless, crystalline residue, after being washed with a little cold water, weighed 5.2 gm. It was recrystallized from 60 cc. of water; yield 3.0 gm. M.p. 187–188° (uncorrected) with decomposition.

Analysis

$C_8H_{12}O_6N_2S_2$. Calculated. N 9.46, S 21.60, neutralization equivalent 148
Found. " 9.41, 9.24, S 21.54, " " 147
$[\alpha]^{25}_{546} = -162.1°$ (1.0 per cent in N NaOH)

Diacetylcystine—Great difficulty has been reported (31, 32) in obtaining pure diacetylcystine. This method, while it does not yield a crystalline product, insures freedom from inorganic salts.

To a suspension of 4 gm. of cystine in 30 cc. of water at 95–100°, 20 cc. of acetic anhydride were added dropwise with stirring over a period of 45 minutes. The solution was allowed to stand overnight, the unchanged cystine (3 gm.) was filtered off, and the filtrate evaporated to a syrup under reduced pressure at 40°. This procedure was repeated with the recovered cystine; the syrups were collected and dried in the oven at 95° for 3 hours. On allowing it to stand at room temperature in a desiccator a brittle mass resulted. The yield was 3.4 gm.

Analysis—$C_{10}H_{16}N_2O_6S_2$. Calculated. N 8.64, S 19.76
Found. " 8.23 " 18.98

For the sulfur lability determinations it was considered to be 95.2 per cent pure. When treated with ethyl alcohol it gave needle-like crystals of acetylcystine ethyl ester, m.p. 122°.

A small amount was dissolved in water and boiled with acid-washed norit for 10 minutes. The colorless filtrate was evaporated under reduced pressure to a syrup and dried at 100°. The slightly yellowish solid had $[\alpha]_D^{24} = -71.1°$ (1.5 per cent in water).

Little racemization occurred during the synthesis; the cystine recovered after hydrolysis by boiling for 3 days with 20 per cent HCl had $[\alpha]_D^{24} = -185°$ (in N HCl).

Dichloroacetylcystine—Prepared by the method of Pirie (33). M.p. 136–138°. $[\alpha]_{546}^{24} = -115.4°$ (3 per cent in N NaOH).

Diglycylcystine—By the method of Pirie (33). M.p. 197–199° (decomposition). $[\alpha]_{546}^{24} = -87.7°$ (0.7 per cent in N NaOH).

Di-α-bromopropionylcystine—By the method of Abderhalden (34). M.p. 145–146.5°. $[\alpha]_{546}^{24} = -93.4°$ (0.7 per cent N NaOH).

Dialanylcystine—By the method of Abderhalden (34). M.p. 206° (browning). $[\alpha]_{546}^{24} = -180.2°$ (0.5 per cent in N NaOH).

Dialanylcystine Anhydride—By the method of Bergmann and Stather (2).

Ditrichloroacetylcystine—To a solution of 4.8 gm. of cystine in 50 cc. of N NaOH, 8.8 gm. of trichloroacetyl chloride and 70 cc. of N NaOH were added in small portions with continuous stirring and cooling below 5°. The addition took about 20 minutes. The mixture was allowed to stand 1 hour at room temperature; it was then acidified with 16 cc. of concentrated HCl, filtered, and extracted with four 25 cc. portions of ethyl acetate. After drying with anhydrous Na_2SO_4, the ethyl acetate was distilled off under reduced pressure till about 10 cc. were left. Ligroin was added; after standing in the ice chest for a week the gummy mass which had precipitated began to crystallize in large needles growing from a center. These were filtered off and recrystallized from ethyl acetate and ligroin. Yield 2.1 gm. M.p. 79–81° (decomposition). $[\alpha]_{546}^{25} = -15.2°$ (2.5 per cent in N NaOH).

Analysis—$C_{10}H_{10}N_2O_6S_2Cl_6$.				
	Calculated.	C 22.09,	H 1.88,	N 5.28
	Found.	" 22.60	" 2.02	" 5.37

Cystine Phenylhydantoic Acid—By the method of Gortner and Hoffman (35). M.p. 148–149°. $[\alpha]_{546}^{25} = -54.1°$ (2.0 per cent in N NaOH).

Cystine Phenylhydantoin—By the method of Shiple and Sherwin (36). M.p. 117–118°.

Dibenzenesulfonylcystine—To a solution of 24 gm. of cystine in 500 cc. of water and 30 cc. of 8 N NaOH, 40 gm. of benzenesulfonyl chloride and 50 cc. of 8 N NaOH were added with stirring. After 3 hours the solution was filtered and glacial acetic acid (22 cc.) was added to incipient precipitation. The solution was decolorized with norit, strongly acidified with 40 cc. of concentrated HCl, and placed in an ice chest. The solid (44 gm., 81 per cent theory) was recrystallized from a mixture of acetone and ethylene chloride. Fine needles, m.p. 213–214°. $[\alpha]_{546}^{24} = +88.2°$ (0.4 per cent in N NaOH).

Analysis—					
$C_{18}H_{20}N_2O_8S_4$.	Calculated.	C 41.54,	H 3.85,	N 5.38,	S 24.61
	Found.	" 41.96,	" 3.98,	" 5.33 (Kjeldahl),	S 24.59
		" 41.99,	" 4.28,	" 5.30 "	" 24.38
				" 5.36 (Dumas)	
				" 5.55 "	

Di-N-methyldibenzenesulfonylcystine—A solution of 5.2 gm. of dibenzenesulfonylcystine in 50 cc. of 2 N NaOH was stirred with 4.9 cc. of dimethyl sulfate; the temperature rose to 35–40°. The clear solution was acidified with dilute H_2SO_4; the oily precipitate, which did not crystallize on long standing at 0°, was repeatedly washed with water, dried, dissolved in a mixture of acetone and $CHCl_3$, filtered, and dried to constant weight.

Analysis—$C_{20}H_{24}N_2O_8S_4$. Calculated. N 5.11, S 23.30
Found (corrected for ash). " 5.16, " 22.68

On boiling this material for 124 hours with a mixture of equal volumes of concentrated hydrochloric and acetic acids, the only crystalline product secured was methylamine benzenesulfonate.

Di-N-benzyldibenzenesulfonylcystine—A mixture of 5.2 gm. of dibenzenesulfonylcystine, 34 cc. of ethyl alcohol, 5 cc. of benzyl chloride, and 6 cc. of 8 N NaOH was boiled gently under a reflux. After 5 minutes boiling, solution was complete; a further 5 cc. of benzyl chloride and 6 cc. of alkali were added, and the boiling was continued for 50 minutes. The alcohol was evaporated off, and the benzyl derivative was thrown out by the addition of acetic acid. The product did not crystallize.

Analysis—$C_{32}H_{32}N_2O_8S_4$. Calculated. N 4.00, S 18.35
Found (corrected for ash). " 3.80, " 16.86

Dibenzylidenecystine—The barium salt was prepared by the method of Bergmann and Zervas (37). For lability determination, an aqueous solution of the salt was treated with the calculated amount of K_2CO_3, filtered, and made up to the standard concentration with NaOH and PbO. The precipitates of PbS were washed with acetic acid to remove any remaining barium before estimation.

Dicarbobenzoxycystine—By the method of Bergmann and Zervas (38).

Oxidized Glutathione—A solution of 0.303 gm. of reduced glutathione in 10 cc. of water previously made slightly alkaline with K_2CO_3 was treated with a trace of $FeSO_4$ and aerated until the nitroprusside test was negative (24 hours). The volume was made up again to 10 cc. and the solution mixed with 10 cc. of plumbite in 2 N NaOH.

Diaminodiethyldisulfide—A sample of the hydrochloride kindly furnished by Dr. R. J. Block had a melting point of 214° (literature, 204°).

Analysis—Cl found, 31.3; calculated, 31.3

Dithiodihydracrylic Acid—By the method of Biilman (39).

In studying the sulfur lability in the cysteine series, the compound was dissolved in plumbite solution which had previously been boiled to remove O_2, and a stream of N_2 was passed through. 5 cc. samples were pipetted into test-tubes (thoroughly washed with N_2) drawn out to a thin neck. The solution was drawn into the lower part of the tube by alternately warming and cooling the tube. The neck was then sealed off and the tubes placed in the thermostat at 25°.

Cysteine HCl—Eastman preparation, m.p. 170–171°.

Acetylcysteine—By the method of Pirie (33). M.p. 104.5°.

Formylcysteine—To a suspension of 1 gm. of diformylcystine in 50 cc. of 5 per cent formic acid, 1 gm. of Zn dust was added. After 12 hours the Zn was filtered off, 5 cc. of 10 per cent H_2SO_4 were added, followed by cuprous oxide. The rest of the procedure was similar to that for acetylcysteine. M.p. 87–89° (effervescence). Yield 0.4 gm. For the I_2 titration, 21.723 mg. required 1.536 cc. of 0.1 N I_2, theory 1.47 cc.

Chloroacetylcysteine and α-Bromopropionylcysteine—By the method of Pirie (33).

Benzenesulfonylcysteine—A solution of 2 gm. of dibenzenesulfonylcystine in 25 cc. of 0.25 N NaOH was stirred for 3 hours with 1 gm. of Zn dust, filtered, and exactly neutralized with dilute HCl. The precipitated $Zn(OH)_2$ was centrifuged off and the solution evaporated under reduced pressure. As the volume decreased, pyramidal crystals separated out. M.p. 136–138°.

Analysis—$C_9H_{11}NO_4S_2$.	Calculated.	C 40.60, H 4.21
	Found.	" 40.49, " 4.31

For the I_2 titration, 10.80 mg. required 0.256 cc. of 0.1 N I_2, theory 0.250 cc.

SUMMARY

1. The introduction of acyl radicals into the amino groups of cystine and cysteine increases the initial lability of the sulfur towards alkali and inhibits the labilizing action of pyruvate. The effect of acylation on the rate of decomposition varies with the negativity of the group. Dibenzenesulfonylcystine is relatively stable towards alkali; its N-alkyl derivatives are less so. Ring

formation markedly increases lability. In diaminodiethyldisulfide, dithiodihydracrylic acid, and homocystine the sulfur is labile to alkali, but to an even smaller degree than with dibenzenesulfonylcystine.

2. The influence of acyl groups on the rate of autoxidation of cysteine derivatives is qualitatively similar to that on the lability towards alkali of the corresponding derivatives of cystine.

3. The sulfhydryl-disulfide system satisfies the requirements for thermodynamic reversibility in the reaction with reversible oxidation-reduction indicators at pH 7 and higher. In all instances studied the potentials have substantially the same value; namely, about 0.23 volt at pH 7. Equilibrium in the reaction between sulfhydryl compounds and dyes in their oxidized form is attained much more slowly than in the reverse direction. The rate of reaction between substituted cysteines and the dyes varies with the character of the substituent.

BIBLIOGRAPHY

1. Clarke, H. T., and Inouye, J. M., *J. Biol. Chem.*, **89**, 399 (1930); **94**, 541 (1931–32).
2. Bergmann, M., and Stather, F., *Z. physiol. Chem.*, **152**, 189 (1926).
3. Brand, E., and Sandberg, M., *J. Biol. Chem.*, **70**, 381 (1926).
4. Nicolet, B. H., *J. Biol. Chem.*, **88**, 395 (1930).
5. Andrews, J. C., and Andrews, K. C., *J. Biol. Chem.*, **102**, 253 (1933).
6. Schöberl, A., *Ann. Chem.*, **507**, 111 (1933).
7. Mathews, A. P., and Walker, S., *J. Biol. Chem.*, **6**, 29 (1909).
8. Thunberg, T., *Skand. Arch. Physiol.*, **30**, 285 (1913).
9. Warburg, O., and Sakuma, S., *Arch. ges. Physiol.*, **200**, 203 (1923).
10. Sakuma, S., *Biochem. Z.*, **142**, 68 (1923).
11. Gerwe, E. G., *J. Biol. Chem.*, **91**, 57 (1931); **92**, 399, 525 (1931).
12. Michaelis, L., and Schubert, M. P., *J. Am. Chem. Soc.*, **52**, 4418 (1930); **53**, 3851 (1931).
13. Schubert, M. P., *J. Am. Chem. Soc.*, **54**, 4077 (1932).
14. Dixon, M., and Tunnicliffe, H. E., *Proc. Roy. Soc. London, Series B*, **94**, 266 (1922).
15. Cannan, R. K., and Knight, B. C. J. G., *Biochem. J.*, **21**, 1384 (1927).
16. Dixon, M., and Quastel, J. H., *J. Chem. Soc.*, **123**, 2943 (1923).
17. Michaelis, L., Oxydation-Reductions Potentiale, Berlin, 2nd edition (1933).
18. Ghosh, J. C., Raychadhuri, S. N., and Ganguli, S. C., *J. Indian Chem. Soc.*, **9**, 43 (1932).
19. Green, D. E., *Biochem. J.*, **27**, 678 (1933).
20. Conant, J. B., *Chem. Rev.*, **3**, 1 (1926).

21. Fruton, J. S., *J. Biol. Chem.*, **105**, 79 (1934).
22. Michaelis, L., *J. Gen. Physiol.*, **16**, 859 (1933).
23. Michaelis, L., and Eagle, H., *J. Biol. Chem.*, **87**, 713 (1930).
24. Clark, W. M., *Pub. Health Rep., U. S. P. H. S.*, **38**, 1669 (1923).
25. Stiehler, R. D., Chen, T., and Clark, W. M., *J. Am. Chem. Soc.*, **55**, 891 (1933).
26. Michaelis, L., *J. Biol. Chem.*, **91**, 369 (1931).
27. Toda, S., *Biochem. Z.*, **172**, 34 (1926).
28. Barron, E. S. G., and Hoffman, L. A., *J. Gen. Physiol.*, **13**, 483 (1930).
29. Kendall, E. C., and Nord, F. F., *J. Biol. Chem.*, **69**, 295 (1926).
30. Dixon, M., and Tunnicliffe, H. E., *Biochem. J.*, **21**, 844 (1927).
31. du Vigneaud, V., and Sealock, R. R., *J. Biol. Chem.*, **96**, 511 (1932).
32. Inoue, Y., *Bull. Inst. Physic. and Chem. Research*, **8**, 697 (1929); *Chem. Abst.*, **24**, 340 (1930).
33. Pirie, N. W., *Biochem. J.*, **25**, 614 (1931).
34. Abderhalden, E., *Fermentforschung*, **9**, 516 (1928).
35. Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, **72**, 433 (1927).
36. Shiple, G. J., and Sherwin, C. P., *J. Biol. Chem.*, **55**, 671 (1923).
37. Bergmann, M., and Zervas, L., *Z. physiol. Chem.*, **152**, 288 (1926).
38. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
39. Biilman, E., *Ann. Chem.*, **339**, 351 (1905).

A ZINC HYDROXIDE POWDER FOR THE PREPARATION OF PROTEIN-FREE FILTRATES OF BLOOD

By T. V. LETONOFF

(*From the Laboratory of the Division of Metabolic Diseases of the Philadelphia General Hospital, Philadelphia*)

(Received for publication, May 1, 1934)

The advantages of colloidal metallic hydroxides as precipitants of blood proteins have been presented by Somogyi (1). Of particular importance in connection with the analysis of the resulting filtrates is the observation that the blood sugar as determined by the commonly used copper reduction methods agrees closely with the true sugar concentration as measured by yeast fermentation procedures. Dry metallic hydroxides (as distinguished from the nascent colloidal material formed in Somogyi's procedure), while frequently used as precipitating agents for proteins in connection with enzyme studies and in other fields, have not been employed for the precipitation of blood proteins. Trial of zinc hydroxide in powder form for this purpose indicated that certain preparations functioned effectively in removing proteins from blood or serum. Not only were the filtrates free from saccharoids so that true sugar values were obtained, but because of the simplicity and convenience of application it appeared that such precipitating reagents might prove quite useful in connection with blood analysis. This would be true particularly where standard solutions are not available or are not readily preserved. Filtrates prepared by the use of a properly prepared zinc hydroxide powder were neutral in reaction, exhibiting remarkable uniformity in this respect. Aside from traces of zinc, easily removed if desired, and small quantities of acetate, added electrolytes did not appear in the filtrates. The reagent may find some use consequently in those instances in which the electrolytes used in existing methods for preparing blood filtrates interfere with the analysis. It was, in fact, such a requirement that led originally to the investigation of zinc hydroxide powder. Advantages offered by the use of this reagent are offset to some extent by its greater cost as compared with the older reagents. On the basis of current prices this is about 0.4 cent per cc. of blood if the material is prepared in the laboratory.

The small amount of zinc present in the filtrates acts as a preservative. As a consequence of this preservative action, filtrates may be kept at room temperature for many days without change in the concentration of sugar or urea. Added to laked blood, zinc hydroxide prevents glycolysis, hence laked blood may be preserved unchanged with respect to these substances simply by the addition of small amounts of the zinc hydroxide powder.

The present paper describes the preparation of a zinc hydroxide powder suitable for use as a precipitant of blood proteins and its application to preparation of protein-free filtrates of whole blood, plasma, serum, and cerebrospinal fluid.

Method

Whole blood (oxalated) is diluted 1:10 with distilled water and is allowed to lake. Approximately 1 gm. of zinc hydroxide powder is added for each cc. of blood. The solution is shaken well for at least ½ minute and allowed to stand for 1 minute. It is then ready for filtration.

Plasma or serum is likewise diluted 1:10 with water. The procedure is the same as for whole blood except that approximately 3 gm. of zinc hydroxide powder are used for each cc. of serum or plasma.

Cerebrospinal fluid, also diluted 1:10, requires only 0.3 gm. of zinc hydroxide powder for each cc. of fluid taken. It is advisable to use a retentive paper for the preparation of filtrates of plasma, serum, or cerebrospinal fluid.

In any of the procedures other dilutions can be substituted if desired, although for most purposes the 1:10 dilution is most convenient. The powder may be measured with sufficient accuracy by means of a calibrated spoon. Filtrates prepared in this way contain traces of zinc. If desired, it may be removed by adding a little sodium carbonate and filtering, although zinc in low concentration does not interfere with determinations of blood sugar, urea, total nitrogen, creatinine, or creatine in whole blood filtrates. Furthermore, because of its preservative action the presence of some zinc in the filtrates is desirable. If urea is to be determined by digestion with urease, a phosphate buffer should be employed, otherwise the urease may precipitate.[1]

The satisfactory preparations of zinc hydroxide were made from zinc acetate and sodium hydroxide. Zinc sulfate failed to give a suitable product. 39.6 gm. of sodium hydroxide in 400 cc. of water are added with stirring to 120 gm. of $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in 1600 cc. of water. After 3 to 5 minutes the precipitate is filtered by vacuum and washed with distilled water until the washings are neutral to phenol red (about 1 liter of water is required). The wet material is dried in air at room temperature on a filter paper or porous plate. Drying is complete when constant weight is reached and when the material can be powdered readily. It is ground to a fine powder in a mortar. The powder that has been used passes through an 80 mesh per inch sieve and is largely retained by 100 mesh. Yields have averaged 53 gm. for the quantities given calculated as $Zn(OH)_2$. A sample exposed to air 5 months in the laboratory retained its original effectiveness as a precipitant.

The powdered material appears to be amorphous, although occasional particles show evidence of crystalline structure. On heating to redness the loss of weight was 28 per cent. Darkening and the appearance of fumes during heating suggested the presence of acetate. Quantitative analysis for acetate of several preparations by distillation in the presence of phosphoric acid (2) demonstrated that it was present uniformly to the extent of slightly more than 10 per cent. Presumably the acetate is present as a basic zinc acetate, in which case the material would consist of zinc hydroxide and basic zinc acetate in the ratio of 4 molecules to 1 molecule. While the data available are not sufficient to permit definite statements in regard to the chemical nature, analytical figures for preparations from different sources are sufficiently consistent to suggest that the material is not a mixture. Principally for convenience, the writer has retained the term zinc hydroxide, although many authorities, it is realized, consider hydrated zinc oxide to be more accurate. Laboratory preparations that deviated from the proportions recommended, as well as ordinary commercial preparations of zinc hydroxide, were found to be unsatisfactory. A suitable zinc hydroxide preparation is now available.[2]

---

[1] Somewhat larger amounts of zinc hydroxide appear in filtrates of plasma or serum than in those of whole blood, and it has been found necessary to treble the usual quantities of phosphate buffer used in the determination of urea in these filtrates. Other buffers than phosphate may be used provided that zinc is removed from the filtrate. The method for accomplishing this is explained in the text. Figures for creatinine in plasma filtrates are slightly lower than in tungstic acid filtrates.

The quantity of reagent used for whole blood protein precipitation can be varied over fairly wide limits without affecting the analytical results. It was found that 0.7 gm. to 1.5 gm. of the zinc hydroxide powder would yield filtrates negative for protein when tested with 10 per cent trichloroacetic acid and saturated picric acid. Less than 0.7 gm. or more than 1.5 gm. gave filtrates containing traces of protein. For plasma or serum, at least 2.5 gm. of zinc hydroxide powder per cc. are required to obtain filtrates free from protein. There appears to be no upper limit in this instance. The procedure as described has been used successfully for precipitating proteins of normal and pathological human blood, as well as that of rabbits and rats. Protein-free filtrates were obtained also from horse serum.

The filtrates seem to keep indefinitely so far as concentration of sugar and urea is concerned. Furthermore, the concentration of sugar in laked blood remains unchanged for many days provided 0.1 to 0.2 gm. or more of zinc hydroxide powder for each cc. of blood is added after the cells are laked. Ordinarily, if the filtration is to be delayed for a few days it is preferable to add 0.2 gm. to the laked blood. The remainder is added at the time of filtration.[3] Zinc sulfate added to blood likewise prevents glycolysis.

Sodium oxalate even in excessive amounts (20 mg. per cc. of blood) does not affect the precipitating action of zinc hydroxide powder. The volume of filtrate obtained is equal to that of tungstic acid filtrates; however, the rate of filtration during the first few minutes is slower when zinc hydroxide powder is used.

---

[2] Manufactured by the J. T. Baker Chemical Company, Phillipsburg, N. J. The writer is indebted to Dr. E. F. Marsiglio for his cooperation in this connection.

[3] When it is mixed with blood, standing overnight at 37° and probably long standing at room temperature appears to denature the zinc hydroxide so that its precipitating action is partially lost. Consequently, if the blood has stood under these conditions with the full amount of precipitant, an additional gm. per cc. must be added just previous to filtration.

The reducing power of protein-free filtrates of diabetic bloods prepared by means of zinc hydroxide powder is compared in Table I with that of similar filtrates prepared according to the Somogyi (1) and Folin and Wu (3) (tungstic acid) procedures. The lowest figures consistently were found in the filtrates from the zinc hydroxide powder precipitation. However, reducing substance in these filtrates was entirely fermentable in contrast to the tungstic acid filtrates which contained considerable amounts of non-fermentable reducing material. Somogyi's procedure and the proposed method gave similar results. Total reduction values in all cases were determined by the Folin and Wu method (4). True glucose of the blood filtrates was determined by fermentation according to the Somogyi (5) procedure, known amounts of glucose being added in the determination of residual reduction after fermentation.

TABLE I

Comparison of Reducing Power of Protein-Free Filtrates of Blood, Prepared by Various Procedures, Determined by Folin and Wu Method

The values are given in mg. of glucose per 100 cc.

Experiment No.	Zinc hydroxide powder			Somogyi's zinc hydroxide			Tungstic acid		
	Total reduction	Saccharoid	Fermentable sugar	Total reduction	Saccharoid	Fermentable sugar	Total reduction	Saccharoid	Fermentable sugar
1	139	0	139	148	6	142	154	15	139
2	152	0	152	152	0	152	168	17	151
3	150	0	150	151	0	151	165	16	149
4	117	0	117	115	0	115	135	17	118
5	80	0	80	82	0	82	95	15	80
6	209	0	209	212	3	209	226	18	208
7	225	0	225	229	4	225	244	19	225
8	110	0	110	110	0	110	125	16	109
9	171	0	171	172	0	172	186	14	172
10	104	0	104	107	4	103	126	23	103
11	51	0	51	50	0	50	74	22	52

Comparison of the concentrations of reducing substances in filtrates of normal bloods prepared by means of the zinc hydroxide powder and tungstic acid showed that in the former the values found were 14 to 25 mg. below those for the tungstic acid filtrates. Similar differences were observed in the filtrates prepared by the two methods from the blood of patients in insulin shock. As described above, these differences are due to the presence of nonfermentable reducing substances in the tungstic acid filtrates, while the values found in the zinc hydroxide powder filtrates represent true sugar.

Benedict (6) has reported that the amount of zinc present in filtrates prepared by Somogyi's method was sufficient to diminish reduction of the Folin and Wu and Benedict's copper reagents for blood sugar. However, the zinc present in whole blood, serum, or plasma filtrates prepared by means of zinc hydroxide powder did not affect the determination of blood sugar by the Folin and Wu method; for results were unchanged when zinc was removed from the filtrates. Addition of zinc sulfate (1 to 5 drops of 1.25 per cent solution) to standard aqueous solutions of glucose, or shaking such solutions with zinc hydroxide powder before determining the reducing power, likewise did not influence the results.

Urea nitrogen (7), creatinine, and creatine (3) determinations in filtrates prepared by the new procedure and the tungstic acid method agree within the limits of experimental error.[1] However, uric acid is completely removed by zinc hydroxide powder and according to Somogyi (8) by his reagent. Coinciding with the observation of this author also, is the lower total nitrogen (3) concentration of filtrates prepared by the proposed method as compared with those prepared by the tungstic acid method. The latter contain 6 to 9 mg. per cent more nitrogen than filtrates prepared by the zinc hydroxide powder method. However, the average normal total nitrogen found by Somogyi in filtrates prepared by his method appears to be significantly lower than in filtrates prepared with the aid of zinc hydroxide powder. Nevertheless, the total nitrogen in the latter seems to parallel that of simultaneously prepared tungstic acid filtrates sufficiently well to enable the results to be interpreted clinically in the same way as nonprotein nitrogen.

SUMMARY

The preparation of a zinc hydroxide powder suitable for precipitation of proteins of whole blood, serum, plasma, and cerebrospinal fluid is described. The resulting filtrates are free of protein, uniformly neutral in reaction, while only small amounts of added electrolytes are present.

Protein-free filtrates prepared by means of this reagent contain no saccharoids and yield true values for reducing sugar. Such filtrates are suitable also for urea nitrogen, creatinine, and creatine determinations. Only traces of uric acid are found. The total nitrogen is lower than in filtrates prepared by tungstic acid precipitation of proteins, but higher than in filtrates prepared by the Somogyi method.

Because of the preservative action of zinc, concentrations of glucose and urea remain unchanged for long periods. Zinc hydroxide powder added to laked blood prevents glycolysis.

The author is indebted to Dr. John G. Reinhold for suggestions and criticism and for assistance with the manuscript.

BIBLIOGRAPHY

1. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
2. Sutton, F., Systematic handbook of volumetric analysis, Philadelphia, 11th edition (1924).
3. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
4. Folin, O., and Wu, H., *J. Biol. Chem.*, **41**, 367 (1920).
5. Somogyi, M., *J. Biol. Chem.*, **78**, 117 (1928).
6. Benedict, S. R., *J. Biol. Chem.*, **92**, 141 (1931).
7. Karr, W. G., *J. Lab. and Clin. Med.*, **9**, 329 (1922)
8. Somogyi, M., *J. Biol. Chem.*, **87**, 339 (1930).

THE INFLUENCE OF THE REMOVAL OF THE PARATHYROID GLANDS ON THE DEVELOPMENT OF RICKETS IN RATS

By JAMES H. JONES

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*)

(Received for publication, July 3, 1934)

The usual changes in the composition of the blood serum which result from the removal of the parathyroid glands are a fall in the concentration of calcium and a rise in the level of inorganic phosphate. In rickets due to low phosphorus content somewhat opposite changes are observed. When a high calcium-low phosphorus rachitogenic diet is fed to rats, there is invariably a fall in phosphate, whereas the calcium remains unchanged or rises slightly above normal. These observations would seem to indicate that either the removal of the parathyroid glands should prevent the development of rickets or a rachitogenic diet should prevent the characteristic blood changes which usually follow parathyroid removal. Pappenheimer (1) has reported that parathyroidectomized rats develop rickets when placed on the Steenbock (2) rickets-producing diet, and Shelling (3) has shown that older rats (6 months of age) on the same diet do not develop parathyroid tetany. In agreement with the lack of tetany the serum calcium and phosphorus were approximately normal. The inorganic phosphate was noticeably above that usually found in young rats which have been on this diet for 2 or 3 weeks. The calcium-phosphorus product was, consequently, considerably higher than the minimum which is thought to be necessary to prevent the development of rickets in rats. Pappenheimer did not report the changes in blood calcium and phosphorus of his experimental animals.

In the following experiments the level of serum calcium and phosphorus in young parathyroidectomized rats on a rickets-producing diet has been studied. Furthermore, the degree to which such animals will develop rickets has been compared with the degree of rickets produced in control animals on the same diet. In addition to the blood studies the degree of calcification was determined by a macroscopic examination of the distal ends of one radius and ulna from each animal according to the line technique, and by ash analysis of the right femur. Simultaneously, similar studies were made on parathyroidectomized animals which were given the Steenbock stock diet (4) without the whole milk supplement.

Although several series of experiments were conducted, the results of only one series will be reported in detail.

Twenty-six animals from five litters and varying in age from 25 to 29 days were placed in individual cages. The parathyroid glands were removed from twenty-two of these animals on the day of segregation. Search was made, in the manner previously described (5), for the glands in the small mass of tissue removed. All animals were then given the dry portion of the Steenbock stock diet from which the $CaCO_3$ was omitted. On this diet parathyroidectomized rats will usually develop tetany within 24 to 48 hours after extirpation of the glands. In this case all twenty-two animals showed definite signs of tetany. Of these animals sixteen were transferred to a rickets-producing diet and the remaining six were given the complete dry portion of the Steenbock stock diet. The transference to these diets was made as soon as definite tetany was observed, which was never more than 48 hours after ablation of the glands. The four animals from which the parathyroid glands were not removed were also placed on the rachitogenic diet, which is a modification of the Steenbock and Black Diet 2965 (2), in which the 76 parts of ground yellow corn were replaced with 66 parts of commercial yellow corn-meal and 10 parts of wheat embryo. This diet contains about half the amount of phosphorus present in the Steenbock diet, and under the conditions prevailing in our laboratory produces rickets in rats with greater regularity. Two of the parathyroidectomized animals (one from the group receiving the rachitogenic diet and one from the group receiving the stock diet) died within a few days. After 24 days on the experimental diets the remaining animals were bled to death. The sera were pooled as indicated in Table I and analyzed for calcium and inorganic phosphorus. The phosphorus was determined on the calcium-free filtrate according to the method of Gunther and Greenberg (6). As seen from Table I the results of both bone and blood analyses indicate that the parathyroidectomized animals on the rachitogenic diet were fully as rachitic as the animals from which the glands were not removed. The diet appears to have completely changed the blood picture from one of parathyroid deficiency to that of low phosphorus rickets. The percentage ash in the femora of the animals from which the para-

TABLE I

Influence of Removal of Parathyroid Glands on Level of Serum Calcium and Phosphorus and on Percentage of Femur Ash of Rats

	Rat No.	Femur ash		Serum	
		Weight		Calcium	Phosphorus
		mg.	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*
Group I. Parathyroidectomized rats on rachitogenic diet	1	28.8	29.7	11.3	2.1
	2	28.7	29.7		
	3	20.3	26.6		
	4	24.5	28.4	12.5	2.2
	5	24.3	27.8		
	6	16.7	23.5		
	7	21.4	29.2	10.9	2.5
	8	18.4	25.6		
	9	16.8	25.5		
	10	35.0	36.5	11.5	2.2
	11	17.6	24.3		
	12	22.1	28.6		
	13	31.8	32.5	12.1	2.5
	14	18.6	25.8		
	15	21.6	26.7		
Group II. Non-parathyroidectomized rats on rachitogenic diet	16	40.2	38.2	12.0	2.7
	17	24.4	30.1		
	18	21.8	29.1		
	19	27.9	30.9		
Group III. Parathyroidectomized rats on stock diet	20	98.4	55.0	7.7	16.4
	21	111.3	54.9		
	22	100.2	54.8		
	23	99.5	53.1	7.9	15.6
	24	116.9	53.8		

thyroids were removed was no higher than that of the control group. In fact, in this experiment the average ash content of the femora of the animals lacking their parathyroids was slightly but definitely below that of the group which retained their parathyroid glands. Examination of the distal ends of the bones of the forearm showed a pronounced rachitic condition in both groups of animals. No difference between the two groups was detectable in this respect. Similarly there was no noticeable difference in the growth of the animals of the two groups.

The results obtained from the parathyroidectomized animals which received the stock diet were, as expected, entirely different. Blood analyses showed the serum calcium to be considerably below normal and the phosphorus considerably above normal. The calcium was just slightly above the tetany level. In spite of this abnormal calcium and phosphorus concentration in the blood these animals grew much better than those of either group on the rickets-producing diet and their general appearance was much better. As Table I shows the bone ash percentage was high, it having increased from approximately 40 per cent to 54 per cent in a little over 3 weeks. The ends of the long bones were also well calcified. These findings are in agreement with our earlier work (5) in which it was shown that if rats are fed a good ration with plenty of calcium the removal of the parathyroid glands has little effect on the rate of bone growth and development, although the serum calcium remains below normal.

In addition to the above experiments, rats which had already developed rickets were parathyroidectomized and continued on the rachitogenic ration. Subsequent examination of the long bones showed no signs of healing of the rickets. These animals did not develop tetany. Results from other similarly conducted experiments, the details of which will not be reported, were in agreement with those given above.

SUMMARY

Young rats from which the parathyroid glands have been removed, subsequently given a high calcium-low phosphorus rachitogenic diet, develop rickets as readily as rats from which the parathyroid glands have not been removed. The concentrations of serum calcium and inorganic phosphorus are those of typical

rickets and the percentage ash of the femora is as low as that in non-parathyroidectomized rats which have subsisted on the same diet for an equal length of time.

Rats from which the parathyroid glands have been extirpated and which were given the dry portion of the Steenbock stock diet show a low blood calcium and high blood phosphorus, but grow at a fairly rapid rate and show a normal increase in percentage of bone ash.

Removal of the parathyroid glands from rachitic rats does not produce any sign of healing of the rickets.

BIBLIOGRAPHY

1. Pappenheimer, A. M., *J. Exp. Med.*, **52**, 805 (1930).
2. Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 263 (1925).
3. Shelling, D. H., *J. Biol. Chem.*, **96**, 195 (1932).
4. Steenbock, H., *Science*, **58**, 449 (1923).
5. Asher, D. W., and Jones, J. H., *J. Biol. Chem.*, **100**, 333 (1933).
6. Gunther, L., and Greenberg, D. M., *J. Biol. Chem.*, **82**, 551 (1929).

STUDIES ON DIGESTIBILITY OF PROTEINS IN VITRO

VI. SOME PARTIAL CLEAVAGE PRODUCTS FROM PEPTIC DIGESTS OF CASEIN

By D. BREESE JONES and CHARLES E. F. GERSDORFF

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington)

(Received for publication, June 19, 1934)

It was recently shown by the authors (1) that when peptic digests of casein were tested for cystine at different intervals by the method of Folin and Marenzi (2) there was a rapid rise in value, reaching a maximum of 0.40 per cent at the end of 1 hour's digestion. On continued digestion the value had dropped to 0.25 per cent by the end of 6 hours. Thereafter it remained constant up to 18 hours. With acid hydrolysis the rise in value was even greater and sharper, reaching 0.55 per cent in 45 minutes. This rise in value was then followed within 15 minutes by an abrupt drop to 0.43 per cent. On further digestion the value gradually decreased, reaching 0.33 per cent by the end of 18 hours, and thereafter remaining constant up to 24 hours.

Inasmuch as it was shown that these values did not represent free cystine, their rise and fall were attributed to the effect of some compound or compounds other than cystine, or to the presence of certain reactive groups in the casein molecule which were exposed during the initial stages of the digestion, and which after reaching a maximum value were rapidly converted into non-reactive compounds or groups.

The interesting observations referred to indicated that far reaching changes in the protein had taken place at a very early stage in the process of proteolysis. The results suggested that a study of the properties and composition of the cleavage products isolated at the time when the digests showed the maximum chromogenic values might be of interest in yielding information on the nature of the changes that occur during the early stages of the digestion and which might throw some light on the structure of the casein molecule.

In this paper are described the isolation, properties, and composition of three fractions obtained by digesting casein with pepsin for 1 hour.

Striking differences in the amino acid composition of the fractions were observed. Fractions A and B, which together represented 34.6 per cent of the casein, contained no cystine. Practically all the cystine of the original casein was recovered in Fraction C. On the other hand, 87 per cent of the phosphorus of the casein was recovered in Fractions A and B. Other differences, although less striking, were observed in the percentages of other amino acids, particularly in those of lysine and tryptophane.

The properties and composition of Fraction A correspond in some particulars to those of certain partial cleavage products of casein which have been previously described and generally referred to as phosphopeptone.

EXPERIMENTAL

Material—The casein used in these studies was prepared from fresh skim milk according to the method of Van Slyke and Baker (3). It was practically free from ash, and contained 15.98 per cent nitrogen and 1 per cent phosphorus.[1]

Fairchild Brothers and Foster's pepsin was used in the digestion work.

Digestion—A quantity of casein calculated to represent 30 gm. of the ash- and moisture-free protein was suspended in a mixture of 375 cc. of 0.1 N hydrochloric acid and 375 cc. of a 0.2 per cent solution of pepsin in 0.1 N hydrochloric acid, the ingredients having been previously heated separately to 38°. The warm mixture was then placed in an incubator and maintained for 1 hour at 38°. At the end of the digestion period the digest was heated to 80° to inactivate the enzyme, and then immediately cooled.

When the prewarmed casein and the pepsin-hydrochloric acid solution were mixed, the former soon assumed a translucent, gelatinous character. Soon after the digestion had started most of the material dissolved, leaving some flocculent, translucent particles in suspension. Under the conditions of the experiment the quantity of the material in suspension increased as the digestion proceeded, and reached its maximum formation at about the end of an hour.

---

[1] Percentages given in this paper have been calculated on the basis of ash- and moisture-free protein.

Fraction A—The flocculent suspension present in the digest was readily separated by centrifugation. After decanting the supernatant liquid, the precipitate was stirred in the centrifuge cup with distilled water until a fine suspension was obtained, and the mixture was again centrifuged. The washing of the product in this manner was repeated five times. It was then similarly washed several times with 95 per cent alcohol, and was finally dried by treatment first with absolute alcohol and then with ether. This preparation, designated Fraction A, consisted of a fine, white powder which amounted to 21.9 per cent of the casein taken for digestion. It contained 15.29 per cent nitrogen and 2.56 per cent phosphorus.

Fraction B—The original supernatant liquid decanted from Fraction A, the aqueous washings, and the first two alcoholic washings were combined, and the total adjusted to pH 6 by addition of dilute sodium hydroxide solution, it having been determined by preliminary tests that the maximum precipitation occurred at this pH. The finely divided material which precipitated was separated by centrifugation, and was washed and dried in the manner described for Fraction A, except that the water used for washing had been brought to pH 6 by addition of acetic acid. The white powder thus obtained, designated Fraction B, represented 12.7 per cent of the casein used for the digestion. It contained 15.25 per cent nitrogen and 2.45 per cent phosphorus.

Fraction C—The centrifugate from Fraction B was combined with the subsequent washings, and the volume was reduced to about 200 cc. by distillation under diminished pressure. The liquid was then transferred quantitatively to a 250 cc. volumetric flask, and the volume brought up to the mark with distilled water. The difference between the weight of the casein taken for the digestion (30 gm.) and the combined weights of Fractions A and B was 19.62 gm., which represents 65.4 per cent of the casein. This fraction is hereafter referred to as Fraction C. Calculated on the basis of 19.62 gm. as the weight of this fraction, the material contained 16.23 per cent nitrogen and 0.06 per cent phosphorus. In the calculation of these figures no account has been taken of the elements of water which may have entered into the reaction as the result of hydrolysis. However, it is not believed that this has any significant effect upon the accuracy of the analytical values given. The fractions studied represent products formed at a very early stage of digestion, probably before any appreciable severance of peptide linkages had occurred, and involving little, if any, hydrolysis.

TABLE I

*Distribution of Nitrogen in Fractions Separated at End of 1 Hour's Peptic Digestion of Casein (Expressed in Percentages of Total Nitrogen)**

Nitrogen	Fraction A	Fraction B	Fraction C	Casein
	per cent	*per cent*	*per cent*	*per cent*
Amide N	12.10	10.56	11.29	11.46
Humin " adsorbed by lime	0.74	0.89	2.46	1.03
" " in amyl alcohol-ether extract	0.09	0.20	0.15	0.16
Arginine N	7.95	8.67	10.87	11.47
Cystine N	0.76	0.89	0.82	0.89
Histidine N	4.98	5.35	7.21	6.01
Lysine N	10.05	13.43	5.34	6.18
Amino N of filtrate	60.39	55.82	22.48	59.64
Non-amino N of filtrate	4.37	5.24	38.65	2.25
Total N regained	101.43	101.05	99.27	99.09

* Nitrogen figures are corrected for the solubilities of the phosphotungstates of the bases. They represent the average results of closely agreeing duplicate determinations.

Distribution of Nitrogen in Fractions A, B, and C—Duplicate samples of Fractions A, B, and C, representing respectively 3.0, 2.0, and 3.1079 gm. of the ash- and moisture-free substances were hydrolyzed by boiling for 30 hours with 60 cc. of 20 per cent hydrochloric acid. The distribution of nitrogen in the hydrolysates was made according to the Van Slyke method. In order to have corresponding data on the casein which was used, for the purpose of comparison, analyses were similarly made on duplicate samples of 3 gm. of the casein, each containing 0.4794 gm. of nitrogen. The results of the analyses, expressed in percentages of the total nitrogen, are given in Table I.

Amino Acids in Fractions—The percentages of amino acids in Fractions A, B, and C, and in the casein are given in Table II. The percentages of nitrogen and phosphorus are also included. The figures for arginine, histidine, and lysine were calculated from the results obtained by the Van Slyke analyses for distribution of nitrogen as given in Table I.

Tryptophane was determined by the method of May and Rose (4), slightly modified as previously described (5).

The determination of cystine was made by the Sullivan method (6), and that of tyrosine by the method of Folin and Ciocalteu (7).

TABLE II

Amino Acid Content of Fractions from Peptic Digest of Casein (Percentages Corrected for Moisture and Ash in Proteins)

	Fraction A	Fraction B	Fraction C	Casein	Method
	per cent	*per cent*	*per cent*	*per cent*	
Cystine	0.00	0.00	0.48	0.33	Sullivan
Arginine	3.78	4.11	5.48	5.70	Van Slyke
Histidine	2.81	3.01	4.32	3.54	"
Lysine	8.02	10.68	4.52	5.15	"
Tyrosine	4.78	5.59	8.76	7.49	Folin-Ciocalteu
Tryptophane	0.46	1.17	3.03	2.23	May-Rose
Nitrogen	15.29	15.25	16.23	15.98	
Phosphorus	2.56	2.45	0.06	1.00	

The most striking differences found in the composition of the three fractions lie in their cystine and phosphorus content. Although Fractions A and B gave positive tests for sulfur by the sodium nitroprusside reaction, no trace of cystine could be detected in these fractions. Practically all the cystine contained in the casein taken for the digestion was recovered in Fraction C. On the other hand, over four-fifths of the phosphorus of the casein was found in Fractions A and B.

Other marked differences, although less striking, differentiate Fraction C from Fractions A and B, particularly the percentages of tryptophane, tyrosine, and lysine.

Although Fractions A and B are very similar with respect to their content of cystine, arginine, histidine, nitrogen, and phosphorus, the differences found in the values for lysine, tyrosine, and particularly tryptophane are believed to justify the conclusion that these two fractions are different substances. It is not unlikely that Fraction B consists of a mixture of several substances precipitated together from the filtrate from Fraction A at pH 6. The properties of Fraction A as well as its behavior on formation and separation indicate that it represents a definite cleavage product.

TABLE III

Showing Distribution of Amino Acids of Casein Among Three Digestion Fractions (Digestion Period, 1 Hour)

	Fraction A	Fraction B	Fraction C	Total recovery
	per cent	*per cent*	*per cent*	*per cent*
Cystine	0.00	0.00	95.13	95.13
Arginine	14.52	9.16	62.88	86.56
Histidine	17.38	10.80	79.81	107.99
Lysine	34.10	26.34	57.40	117.74
Tyrosine	13.98	9.48	76.49	99.95
Tryptophane	4.52	6.66	88.86	100.04
Phosphorus	56.06	31.12	3.92	91.10

TABLE IV

Color Values on Fractions A, B, and C Expressed in Terms of Cystine As Determined by Folin and Marenzi Method

Fraction	Hydrolyzed	Unhydrolyzed
	per cent	*per cent*
A	0.149	0.17
B	0.38	0.68
C	0.323	0.40

Table III shows how the amino acids and phosphorus of the casein were distributed between the three digestion fractions. It is of interest to note that, besides cystine, practically 95 per cent of which was recovered in Fraction C, 80 per cent of the histidine, 76.5 per cent of the tyrosine, and nearly 89 per cent of the tryptophane of the casein were also found in this soluble fraction, but only 4 per cent of the phosphorus.

Cystine determinations were made on Fractions A, B, and C also by the Folin and Marenzi method, both after the fractions had been hydrolyzed by boiling with 20 per cent hydrochloric acid for 18 hours and also on the fractions without first subjecting them to hydrolysis. For the determinations on the unhydrolyzed material, Fractions A and B were first dissolved in dilute sodium carbonate solution and the solution was slightly acidified with hydrochloric acid. The resulting finely divided suspension was diluted with distilled water to a definite volume and uniform samples were tested according to the Folin and Marenzi method. The aqueous solution of Fraction C was tested directly. The results are shown in Table IV.

The sum of the chromogenic values found for the unhydrolyzed fractions when calculated on the basis of the proportionate quantities of the fractions isolated from the casein amounted to a cystine-equivalent value of 0.39 per cent. This value agrees closely with that previously found (1) in the total peptic digest at the end of 1 hour's digestion. As pointed out, these values do not represent free cystine, but are to be ascribed to the effect of some compound or compounds other than cystine, or to that of certain reactive groups in the casein which are exposed during the early stages of the digestion.

The determinations made on the hydrolyzed fractions gave a combined value, similarly calculated, of 0.29 per cent. This value is in fairly close agreement with the cystine content of the casein used for the digestion.

A number of investigators (8–11) have described the separation and properties of partial cleavage products formed when casein is digested with pepsin or trypsin. Some of these products correspond rather closely with our Fraction A. They have been variously termed "paranuclein," "pseudonuclein," "phosphopolypeptide," "lactotyrine," and "phosphopeptone."

Posternak (12) reported the isolation of a product from a peptic digest of casein which on hydrolysis gave glutamic acid, aspartic acid, isoleucine, serine, and phosphoric acid.

Rimington (13) obtained by tryptic digestion of casein a phosphopeptone which on hydrolysis with 20 per cent hydrochloric acid yielded products which had the composition and properties of hydroxyglutamic acid, hydroxyaminobutyric acid, serine, and phosphoric acid. He was unable to detect the presence of any other amino acid. On the basis of this analysis he constructed a hypothetical structural formula for the phosphopeptone.

Holter, Linderstrøm-Lang, and Funder (14) point out that the special character of peptic casein digestion lies in the temporary separation of an intermediate product, phosphopeptone. They observed that during peptic digestion of casein there is at first a decrease in viscosity, then an increase to a maximum which coincides with the separation of a coagulum in the digestion mixture. The viscosity maximum is due to the separation of an insoluble phosphopeptone, separating first as a gel, which soon flocculates. By treating casein for 10 to 15 minutes with successive portions of 60 per cent alcoholic hydrochloric acid (0.002 N to 0.001 N hydrochloric acid) at 60–70°, they obtained three fractions, one of which, insoluble in the reaction mixture and high in phosphorus content, had properties similar in some respects to the phosphopeptone formed from casein by the action of pepsin as described by them.

Levene and Hill (15) isolated a phosphopeptone (dipeptide) from a tryptic digest of casein. On hydrolysis with hydrochloric acid it yielded glutamic acid, serine, and phosphoric acid. A similar product was obtained by Schmidt (16), but which also contained leucine.

Grabar (17) obtained from a tryptic digest of casein a product precipitable by trichloroacetic acid, having 13.1 per cent nitrogen. A test for tryptophane gave negative results, but the Millon test showed the presence of a trace of tyrosine.

Inasmuch as the phosphopeptone is decomposed by further action of pepsin, and its composition is continually changing, direct comparison of the products described cannot be made, nor is it at all likely that any one of them is identical with another. Some of the products described as having only a few amino acids are apparently much simpler than Fraction A described in this paper. They doubtless represent products of a more advanced stage of hydrolysis. It is of interest to point out a certain correlation between the composition of the fractions separated by Linderstrøm-Lang (18) by the action of alcoholic hydrochloric acid and that of Fractions A, B, and C described in this paper. Those which contained the most phosphorus had also the highest content of tryptophane, tyrosine, histidine, and arginine.

The ease with which casein can be resolved into various fractions differing characteristically from one another, such as those described in this paper, strongly supports Sörensen's conception that soluble proteins represent reversibly dissociable component systems. It has been shown by Sörensen (19), Linderstrøm-Lang (18), Svedberg, Carpenter, and Carpenter (20), and others that casein is not an individual protein but an aggregation of several molecular components or complexes forming a coprecipitation system. According to Sörensen, these complexes, mainly polypeptides, are loosely and reversibly bound by means of residual or secondary valencies to form the whole protein component system, while the atoms or atom groups within each complex are firmly linked together by main or primary valencies.

The early stage of the digestion at which Fraction A began to separate from the digest strongly suggests that it may be regarded as representing one of the complexes of the casein molecular system in accordance with Sörensen's conception.

The manner of separation and the results of analyses of Fraction A show that this phosphopeptone is at least partly responsible for the high and increasing color values found in the casein peptic digest during the 1st hour's digestion by Folin and Marenzi's method for estimating cystine. The rapidly rising values, reaching a maximum at the end of the 1st hour and then decreasing on further digestion, closely correlate with the observations noted that the phosphopeptone begins to separate at an early stage of the digestion and reaches its maximum formation at about the end of 1 hour, thereafter decreasing in amount as it becomes acted upon itself by the pepsin.

SUMMARY

Three cleavage products of casein have been isolated after 1 hour's digestion with pepsin. Fraction A represents the flocculent, translucent material which gradually separated during the digestion. Fraction B was obtained by adjusting the filtrate from Fraction A to pH 6. The products remaining in the solution after removal of Fraction B represent Fraction C. The three fractions amounted, respectively to 21.9, 12.7, and 65.4 per cent of the casein taken for the digestion.

Striking differences in the amino acid composition of the fractions are noted. Fractions A and B contained no cystine. Frac-

tically all the cystine of the original casein was accounted for in Fraction C. On the other hand, 87 per cent of the phosphorus of the casein was accounted for in Fractions A and B.

Other differences, although less striking, are noted in the percentages of other amino acids, particularly of lysine and tryptophane. The percentages of lysine in Fractions A, B, and C were 8.02, 10.68, and 4.52, respectively, and those of tryptophane, 0.46, 1.17, and 3.03.

Fraction A corresponds in some particulars to partial cleavage products of casein previously described, and generally referred to as phosphopeptone.

BIBLIOGRAPHY

1. Jones, D. B., and Gersdorff, C. E. F., *J. Biol. Chem.*, **101**, 657 (1933).
2. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
3. Van Slyke, L. L., and Baker, J. C., *New York Agric. Exp. Stat., Tech. Bull. 65* (1918).
4. May, C. E., and Rose, E. R., *J. Biol. Chem.*, **54**, 213 (1922).
5. Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, **62**, 192 (1924-25).
6. Sullivan, M. X., *Pub. Health Rep., U.S.P.H.S.*, suppl. 78, 6 (1929).
7. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
8. Alexander, J., *Z. physiol. Chem.*, **25**, 411 (1898).
9. Plimmer, R. H. A., and Bayliss, W. M., *J. Physiol.*, **33**, 439 (1905-06).
10. Reh, A., *Beitr. chem. Physiol. u. Path.*, **11**, 1 (1907).
11. Solkowski, E., *Z. physiol. Chem.*, **27**, 297 (1899); **32**, 245 (1901).
12. Posternak, M. S., *Compt. rend. Acad.*, **184**, 306 (1927).
13. Rimington, C., *Biochem. J.*, **21**, 204, 272, 1179, 1187 (1927).
14. Holter, H., Linderstrøm-Lang, K., and Funder, J. B., *Compt.-rend. trav. Lab. Carlsberg*, **19**, No. 10 (1933).
15. Levene, P. A., and Hill, D. W., *J. Biol. Chem.*, **101**, 711 (1933).
16. Schmidt, G., *Z. physiol. Chem.*, **223**, 86 (1934).
17. Grabar, P., *Compt. rend. Soc. biol.*, **114**, 13 (1933).
18. Linderstrøm-Lang, K., *Compt.-rend. trav. Lab. Carlsberg*, **17**, No. 9 (1929).
19. Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, **18**, No. 5 (1930).
20. Svedberg, T., Carpenter, L. M., and Carpenter, D. C., *J. Am. Chem. Soc.*, **52**, 241, 701 (1930).

THE LIPIDS OF MILK

I. THE FATTY ACIDS OF THE LECITHIN-CEPHALIN FRACTION

By FLOYD E. KURTZ, G. S. JAMIESON, and GEORGE E. HOLM

*(From the Research Laboratories, Bureau of Dairy Industry, and the Oil,
Fat and Wax Laboratory, Bureau of Chemistry and Soils, United
States Department of Agriculture, Washington)*

(Received for publication, June 21, 1934)

Phospholipids, occurring as they do in all cells, are considered of great biological importance. In 1919, Meigs, Blatherwick, and Cary (1) showed that the blood leaving the mammary gland contained more inorganic phosphorus and less lipoid phosphorus, by a comparable amount, than did that entering the gland. This observation, by leading to the hypothesis that these compounds are intermediaries in the synthesis of milk fat, has given an additional interest to their study.

The most important variation in the composition of phospholipids from different animal sources is, in general, in the nature of their component fatty acids. Burow (2) and others, without investigating the nature of the fatty acids, developed methods for determining the phospholipid content of milk. Later work by Schlossmann (3) and by Njegovan (4) questioned the existence of phospholipids in milk. The work of Osborne and Wakeman (5) established the fact that mixed phospholipids are present in milk in small quantities. They identified a monoamino-, and, presumably, a diaminophospholipid. Their data indicated the presence of oleic and stearic acids in the monoamino fraction.

Sasaki and Hiratsuka (6) concluded that the lecithin of milk contains myristic and lauric acids, and the cephalin of milk, palmitic and lauric acids.

These investigators were handicapped by dealing with such small amounts of material that accurate analyses were extremely difficult.

Preparation of Material

The present investigation was made upon the material obtained from the extraction of 300 pounds of sweet cream, spray-dried, buttermilk powder. This powder, in smaller lots, was slowly sifted into a mixture of 3 parts of ethanol and 1 part of gasoline, contained in a glass-lined tank. During the addition the contents of the tank were stirred continuously by means of a centrifugal pump. This procedure was important in preventing the powder from caking. Each batch of powder was left in contact with the solvent for about 24 hours. The powder was then removed from the solvent by means of a basket centrifuge. After concentration in a vacuum pan, the solution of lipids was added to an excess of acetone. The precipitated phospholipids were taken up in ether and reprecipitated with acetone. This process was repeated about ten times. The material was then again taken up in ether and the solution decanted from the insoluble residue.

After precipitation with acetone, the phospholipids were emulsified with water, reprecipitated, and then triturated with hot acetone. This last process was carried out for the purpose of removing any saturated fats that might be present (7). An ether solution of the phospholipids was then held overnight at a temperature of 0°. The solution was decanted from the precipitate, the dissolved material precipitated with acetone, redissolved in ether, and the solution recooled. This procedure was repeated until no more precipitate was formed when the ether solution was held at 0°.

The ether-insoluble material so obtained weighed 95 gm. After purification by crystallization from ethanol and subsequent washing with ether, 60 gm. of a white, powdery material remained. Analytical values indicated this to be a mixture of about equal quantities of sphingomyelin and galactolipids. The results of an analysis of this material will be reported later.

The ether-soluble material obtained at this point weighed 580 gm. A titration with potassium hydroxide (8) indicated that it was a mixture of 35 per cent cephalin and 65 per cent lecithin. It contained 3.70 per cent P and 1.97 per cent N, which indicated the presence of nitrogenous impurities.

This material was dissolved in ether, added to a large excess of absolute alcohol, and the solution cooled to −18°. The bulk of the material separated.

Further purification of this alcohol-insoluble fraction by means of the cadmium chloride salt (9) was unsuccessful because nearly all of the salt remained soluble in ether. Other attempts to lower its nitrogen content failed until it was reemulsified with water. After twice emulsifying with a large excess of water, and precipitating with acetone, the nitrogenous impurities were removed.

The purified material obtained was used for the analysis of the fatty acids. It contained 3.69 per cent P and 1.61 per cent N, an atomic ratio of P:N of 1.03:1.00. A titration indicated that it was a mixture of 44 per cent cephalin and 56 per cent lecithin. It was a dark brown, soft, hygroscopic material, entirely soluble in ether. It remained soluble in ether even after long standing, either in the cold or at room temperature. It yielded 62.5 per cent of fatty acids upon saponification with alcoholic potassium hydroxide.

Analysis of Fatty Acids

A preparation of acids was secured by boiling the lecithin-cephalin mixture for 5 hours with alcoholic potassium hydroxide in an atmosphere of nitrogen. 120 gm. of these acids were separated by the lead salt-ether method. 30 gm. of saturated and 77 gm. of unsaturated acids were secured. The loss of 13 gm. of acids was entirely of the unsaturated fraction, due to the formation of persistent emulsions.

For the purpose of later calculations, the unsaturated fraction was assumed to weigh 90 gm.

The lead salts of the saturated acids were decomposed with dilute nitric acid. With this treatment no emulsions were formed, and the acids were wholly recovered.

Saturated Fraction—The analysis of the saturated acids was made by methods already described (10, 11). Briefly, the acids were esterified with ethanol and the esters were distilled at about 4 mm. pressure into four fractions. The iodine number and saponification value were determined on each fraction. These data are given in Table I.

The acids of each fraction were recovered and fractionally crystallized from ethanol. The identification of each acid was made on the basis of the isolation of a fraction giving a correct melting point which was unchanged by further recrystallizations and which also was unchanged when the acid was mixed with an equal quantity of the known acid. Fraction 1 consisted of nearly equal quantities of myristic and stearic acids, Fraction 2 mostly of stearic with a little myristic acid, Fraction 3 mostly of stearic with a little arachidic acid, and Fraction 4 of stearic and arachidic acids. Arachidic acid was recovered from the residue.

After correction for the unsaturated acids present, the composition of the saturated acids of this fraction was found to be: 17.4 per cent of myristic acid, 74.1 per cent of stearic acid, and 8.5 per cent of arachidic acid.

TABLE I

Data on Saturated Ester Fractions

Fraction No.	Pressure	Distillation temperature	Weight	Saponification value	Iodine No. (Hanus)
	mm.	*°C.*	*gm.*		
1	2	145–166	4.75	196.1	9.5
2	4	183–185	17.25	184.4	15.0
3	3.5	186–192	5.90	179.7	17.8
4	3	192–210	2.40	169.6	16.7

TABLE II

Data on Unsaturated Ester Fractions

Fraction No.	Pressure	Distillation temperature	Weight	Mean mol. wt.	Iodine No. (Hanus)
	mm.	*°C.*	*gm.*		
1	3 –2.5	165–190	7.60	302.6	81.5
2	2.5–2	190–195	14.20	312.00	94.7
3	2	195	20.80	317.7	100.3
4	2	200–218	6.90	325.2	114.0
Residue			1.20		

Unsaturated Fraction—A portion of the unsaturated acid fraction was esterified and fractionally distilled in the presence of carbon dioxide, with the results shown in Table II.

It was found that Fraction 1 contained myristic acid as was expected, in addition to oleic acid. The other three fractions contained the esters of unsaturated acids only, one ester of which was far more unsaturated than that of oleic acid. No evidence could be found of the presence of any linoleic or linolenic acids.

Oleic Acid—Fraction 1 was saponified and the free acids, dissolved in 70 per cent ethanol, were held for several days at 5°. Myristic acid separated, and was removed. A portion of the acids recovered from the alcohol solution was hydrogenated. Only stearic acid could be found in the hydrogenated product. Another portion of this fraction was oxidized according to the method of Lapworth and Mottram (12). The dihydroxystearic acid formed, melted sharply at 128.5° and was unchanged by repeated recrystallizations from ethyl acetate. As the melting point of this acid is usually reported as 132–133°, it was considered possible that this oleic acid was not the ordinary $\Delta^{9:10}$ oleic acid.

This possibility was tested by examination of Fraction 2 of the unsaturated esters. The analytical values of this fraction indicated that it contained about 95 per cent oleic acid and only 5 per cent of the highly unsaturated acid. 10 gm. of this fraction were oxidized according to the method of Armstrong and Hilditch (13). The monobasic acid formed by the oxidation, being liquid, was present in insufficient quantity for purification and identification. The dibasic acid, however, was purified by crystallization from acetone and from water. Azelaic acid was identified by means of its melting point and neutralization equivalent. Furthermore, no other dibasic acid could be found, each succeeding crop giving analytical values corresponding to those of azelaic acid. So it would appear that the oleic acid found here is also a $\Delta^{9:10}$ acid.

Polyunsaturated Acid—Portions of Fractions 3 and 4 of the unsaturated esters were brominated for the purpose of determining the nature of the polyunsaturated acid or acids present. The presence of tetrabromides or hexabromides could not be established. However, in each case there was secured a bromide insoluble in ethyl ether which upon heating did not melt at above 200°. Analysis of the bromide gave 63.4 per cent of bromine, whereas the calculated value for ethyl octabromobehenate is 63.90 per cent, and for ethyl octabromoarachidate is 65.79 per cent bromine. On the assumption that a dicostetrenoic acid was present, it was calculated from the analytical data that the unsaturated acid fraction contained 1.95 per cent of myristic acid, 8.04 per cent of dicostetrenoic acid ($C_{22}H_{36}O_2$), and 90.01 per cent of oleic acid.

As no evidence of linoleic or linolenic acids could be found, the thiocyanogen value of the original unsaturated acids was determined and found to be 96.8. The iodine number (Hanus) of these same acids was 109.0. From these results it is evident that more than one double bond of the highly unsaturated acid reacted with the thiocyanogen radical. Otherwise, on account of the presence of myristic acid, the value for the thiocyanogen number would have been below that for oleic acid. On the assumption that two double bonds of the dicostetrenoic acid reacted, the thiocyanogen value of this acid would be 152.8, the iodine number being 305.5. With the formula, per cent of dicostetrenoic acid $=$ 0.655 (iodine value $-$ SCN value), it was calculated that the unsaturated acids contained 7.99 per cent of dicostetrenoic acid, which value is in close agreement with that (8.04 per cent) obtained by use of the mean molecular weights of the unsaturated ester fractions. If three double bonds reacted with the SCN radical, the calculated per cent of this acid would be much higher. Therefore, it is evident that but two of the four double bonds of this dicostetrenoic acid react with the thiocyanogen radical.

The authors are indebted to Mr. R. S. McKinney for the thiocyanogen study contained in this paper.

Other evidence that the highly unsaturated acid was a C_{22} acid was that only on this assumption could the values obtained for the mean molecular weight and for the iodine number be harmonized.

The ether-insoluble bromide, referred to above, was debrominated with zinc in ethanol containing a little HCl. The fatty acid was recovered and then hydrogenated. The saturated acid was saponified to decompose any esters present. The free acid was then recovered and crystallized from ethanol. After several recrystallizations the acid melted at 71.5°. This melting point was maintained after recrystallization. The amount of acid remaining was too small for further attempts at purification. When mixed with an equal quantity of stearic acid (m.p. 71.5°) obtained from sapote oil, the melting point was depressed several degrees.

A further attempt was made to isolate the saturated acid corresponding to the highly unsaturated acid. Fraction 3 of the unsaturated esters was saponified and the free acids combined with about 20 gm. of the unsaturated acids which had not been used for esterification. These acids were converted into their lithium salts and crystallized from 50 per cent ethanol. By this method, the highly unsaturated acid was concentrated to about 15 per cent in the soluble fraction. The acids were recovered from their salts and hydrogenated. From the saturated acids obtained, after repeated crystallizations from ethanol, was isolated a product which melted at 71°. The amount of this acid was too small for further examination. Another product isolated from this fraction melted at 63°, and by saponification showed a mean molecular weight of 309. A third product, also melting at 63°, showed a mean molecular weight of 314. This is approximately the value that would be obtained with a 50:50 mixture of stearic and behenic acids. If the unknown acid were of the C_{22} series, it possibly could be an isomeric form, which would have a lower melting point and greater solubility than that of ordinary behenic acid.

It must be concluded that the nature of the highly unsaturated acid was not proved, but the indications of a variety of analytical data were that it is a dicostetrenoic acid ($C_{22}H_{36}O_2$).

After correcting for the myristic acid present, the composition of the unsaturated fraction was calculated to be 91.8 per cent oleic acid, and 8.2 per cent dicostetrenoic acid(?).

The composition of the total acids was found to be as follows:

	per cent
Myristic acid	5.2
Stearic acid	16.1
Arachidic acid	1.8
Oleic acid	70.6
Dicostetrenoic acid (?)	6.3

SUMMARY

In contrast to butter fat, the lecithin-cephalin fraction of the milk phospholipids contains none of the lower fatty acids. More surprising is the entire absence of palmitic acid, which not only is one of the acids most abundant in butter fat, but is widely distributed among a great many fats and oils, both of vegetable and of animal origin.

The high percentage of oleic acid shows that a considerable proportion of the phospholipid molecules contains only unsaturated acids. The indication of a dicostetrenoic acid is in harmony with recent work on the phospholipids from other sources (14).

BIBLIOGRAPHY

1. Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, **37**, 1 (1919).
2. Burow, R., *Z. physiol. Chem.*, **30**, 495 (1900).
3. Schlossmann, A., *Arch. Kinderh.*, **40**, 18 (1904).
4. Njegovan, V., *Biochem. Z.*, **54**, 78 (1913).
5. Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, **21**, 539 (1915).
6. Sasaki, R., and Hiratsuka, E., *Proc. Imp. Acad.*, Tokyo, **7**, 99 (1931).
7. Sueyoshi, Y., *J. Biochem.*, Japan, **13**, 145 (1931).
8. Rudy, H., and Page, I. H., *Z. physiol. Chem.*, **193**, 251 (1930).
9. Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, **46**, 193 (1921).
10. Baughman, W. F., and Jamieson, G. S., *J. Am. Chem. Soc.*, **42**, 156 (1920).
11. Jamieson, G. S., and Baughman, W. F., *J. Am. Chem. Soc.*, **42**, 1200 (1920).
12. Lapworth, A., and Mottram, E. N., *J. Chem. Soc.*, **127**, 1628 (1925).
13. Armstrong, E. F., and Hilditch, T. P., *J. Soc. Chem. Ind.*, **44**, 43 T (1925).
14. Klenk, E., and von Schoenebeck, O., *Z. physiol. Chem.*, **194**, 191 (1931).

FURTHER STUDIES ON THE PROTEOLYTIC ENZYME CONTENT OF LATEX FROM THE FIG AND RELATED TREES*

BY BENJAMIN H. ROBBINS AND PAUL D. LAMSON

(*From the Department of Pharmacology, Vanderbilt University School of Medicine, Nashville*)

(Received for publication, June 14, 1934)

In a former paper (1) it was shown that there is present in the sap of *Ficus laurifolia* a proteolytic enzyme of the tryptic type which we believe is the active anthelmintic principle in the sap. Several workers have shown that the sap is of value as an anthelmintic (2–6) and is widely used as such among natives in Central America and South America.

Because of the capacity of this substance to digest live tissue (*Ascaris*), we were interested in studying the sap from other species of the genus *Ficus* and related genera to find if this activity was generally present in all of the species.

Through the courtesy of Professor Barbour[1] we were able to collect and test sap from several species of the family Moraceæ at Soledad, Cuba. Sap was obtained from sixteen species of the genus *Ficus* and from one species each of four other genera of the family Moraceæ. Several samples of sap from South America were obtained for us by the International Health Division of the Rockefeller Foundation. The results obtained when these specimens were examined for their enzymatic action on gelatin and *Ascaris* are shown in Table I.

Methods Used for Determining Proteolytic Activity—The method

---

* The funds for carrying out this work were given by the International Health Division of the Rockefeller Foundation.

[1] We wish to express our appreciation to Dr. Thomas Barbour, Director of the Harvard Botanical Museum, for the opportunity and facilities afforded us in the collection and analysis of sap at the Harvard Botanical Gardens at Soledad, Cuba.

Action of Sap from Cuba, Alabama, and South America upon Gelatin and Ascaris

	Family	Genus and Species	Increase in amino N by action of 1 cc. of sap on 100 cc. of 2 per cent gelatin solution for 24 hours at 35°	Action on *Ascaris*
			mg. N₂	
1. From Cuba (classification by Mr. Robert M. Grey, botanist at Harvard Botanical Gardens, Soledad, Cuba)	Moraceæ	*Artocarpus foret*	0.0	
		Brosimum alicastrum	7.5	
		Broussonetia papyrifera	10.0	None in 24 hrs.
		Ficus aurea	0.0	
		“ *benghalensis*	0.0	“ “ 24 “
		“ *benjamini*	9.0	“ “ 24 “
		“ *brevifolia*	2.5	
		“ *carpensis*	0.0	
		“ *crassinervia*	1.2	
		“ *elastica*	0.0	
		“ *glabrella*	1.2	
		“ *glamarata*	0.0	
		“ *nitida*	10.0	“ “ 24 “
		“ *nota*	2.5	
		“ *religioso*	0.0	
		“ *spragueana*	1.2	
		“ *vogelii*	0.0	
		“ unknown	24.0	Digested in 24 hrs.
		Morus nigra	2.5	
2. From Alabama		*Ficus carica*, L.	65	“ “ 1½ “
3. From South America (local names)		Higueron hembra	60	“ “ 1½ “
		Ficus glabrata	52	“ “ 2 “
		Higueron liso	50	“ “ 2½ “
		“ rojo	45	“ “ 2½ “
		“ “ hembra	42	“ “ 6 “

of determining quantitatively the action on gelatin is by use of formol titration. To 100 cc. of a 2 per cent gelatin solution is added 1 cc. of the fresh sap. Formol titration is carried out on a 20 cc. aliquot immediately. The mixture is then incubated at 35° for 24 hours and the titration repeated. The difference between the two determinations is due to the hydrolysis of gelatin and is proportional to the proteolytic activity of the sap. Table I gives the action of the sap on gelatin in terms of mg. of amino nitrogen liberated by the action of 1 cc. of sap upon 100 cc. of a 2 per cent solution of gelatin at 35° for 24 hours.

To test the action on *Ascaris*, fresh worms (from the pig) are incubated at 35° in 100 cc. of Ringer's solution to which 2 cc. of the sap have been added. Controls are run by using sap from the *Ficus glabrata* which, when added to the Ringer's solution in 1 to 2 per cent concentration will digest the worms in 1½ to 2 hours.

From Table I it is obvious that the proteolytic activity of all specimens from Cuba except *Ficus*, unknown, is essentially negative.

The sap was collected during the middle of November, and one might attribute the relative lack of enzyme to seasonal variation, except for the fact that *Ficus*, unknown, was moderately high in enzyme content and that *Ficus carica*, L., was very high. However, the sap of *Ficus carica*, L., was not obtained from Cuba, but from southern Alabama during the same week that the others were collected. The enzyme content of the sap from South America is quite high.

SUMMARY

1. Sap from sixteen species of the genus *Ficus* has been studied and a proteolytic enzyme found in three cases, but only in two was the concentration high enough to digest *Ascaris* in 2 per cent solution of sap.
2. Sap from four genera of the family Moraceæ has been studied for proteolytic activity. The activity was less than one-fifth of that noted in sap from *Ficus carica*, L., collected at the same time.
3. Sap from six trees in South America has been examined and five samples were high in proteolytic enzyme.

BIBLIOGRAPHY

1. Robbins, B. H., *J. Biol. Chem.*, **87**, 251 (1930).
2. Caldwell, F. C., and Caldwell, E. L., *Am. J. Trop. Med.*, **9**, 471 (1929)
3. Hall, M. C., and Augustine, D. L., *Am. J. Hyg.*, **9**, 602 (1929).
4. Mouat-Briggs, C. E. F., *Tr. Roy. Soc. Trop. Med. and Hyg.*, **8**, 21 (1914-15).
5. Paez, F., *Tr. Roy. Soc. Trop. Med. and Hyg.*, **8**, 217 (1914-15).
6. Montoya, T. W., *Trop. Dis. Bull.*, **19**, 240 (1922).

THE DIFFERENTIATION OF TRYPSINS BY MEANS OF THE ANAPHYLACTIC TEST

BY CARL TEN BROECK

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton*)

(Received for publication, May 25, 1934)

Kunitz and Northrop (1) have recently reported the crystallization of chymotrypsinogen and chymotrypsin and have shown that the latter differs from the trypsin that they had studied previously. The present work was undertaken to determine whether these substances could be differentiated by immunological reactions. Trypsin from the pig and cow as well as chymotrypsinogen and chymotrypsin from the cow were used.

The studies on the antigenic nature of enzymes made before these substances were purified by crystallization are all open to question, for it is impossible to determine whether the reactions obtained were due to the enzymes as such or to the proteins with which they were associated. Northrop (2) found that the serum of a rabbit immunized to crystalline pepsin precipitated this enzyme when it was diluted to 1:2000. This immune serum had a greater antipeptic action than did the serum from a normal rabbit or from a rabbit immunized to the pepsin denatured by heat. Sumner and Kirk (3) have shown that their crystalline urease was antigenic, for they produced in rabbits an antiurease that *in vitro* and *in vivo* neutralized the action of the enzyme. Kirk (4) was also able to cause the production of a precipitin that he has used in purifying urease. Kirk and Sumner (5) were unable to differentiate by the precipitin or the antiurease reaction the urease obtained from the soy or jack bean.

The essential part of this work, namely the preparation of the enzyme solutions, was done by Drs. Northrop and Kunitz. The enzymes have been recrystallized at least five times and were kept in concentrated, slightly acid solution in the cold. Fresh dilutions of known concentration were prepared for each test.

The anaphylactic test rather than the less sensitive precipitin reaction was used in the major portion of the tests for differentiation since the enzymes may be very closely related.

Sensitization of Guinea Pigs—Female guinea pigs weighing about 125 gm. were given subcutaneous injections of 0.5 cc. of either 0.5 or 1 per cent solution of the enzymes. Injection of chymotrypsinogen produced no visible effect, but the animals receiving the trypsin and the chymotrypsin showed necrotic areas at the site of inoculation and several died. It was our impression that the chymotrypsin was more toxic than the trypsin.

TABLE I

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Pig Trypsin

In this and in Tables II to IV L and R indicate left and right horns of the uterus. A = first test; B, second; etc. Figures are the concentration of the enzymes in the bath in mg. of protein per ml. + or − indicates contraction or failure to contract.

Guinea pig No.	Reaction to		
	Pig trypsin	Beef trypsin	Chymotrypsin
1	RB 10^{-4} −	RA 10^{-3} −	
	RC 10^{-3} +		
	LA 10^{-4} −		
	LB 10^{-3} +		
2	LB 10^{-3} +	LA 10^{-3} −	
	RB 10^{-3} +		RA 10^{-3} −

One guinea pig was not sensitive.

Tests for Sensitization—Between 15 and 20 days after the first injection, the uteri of these guinea pigs were tested by means of the Dale technique (6). Two baths were used and the two horns of the uterus were tested, one after the other. The capacity of each bath was 75 cc., and 0.75 cc. of the enzyme solution was added for each test.

The results of the tests are given in Tables I to IV. Not all of the animals were sensitized, and in some cases there were cross-reactions, particularly between the chymotrypsin and chymotrypsinogen. The results were, however, sufficiently clear cut to show that all four of these enzymes can be differentiated by this reaction.

Tests for Precipitin—The undiluted serum from a rabbit that had received repeated injections of chymotrypsin was layered under dilutions of this enzyme. In 1 per cent and 0.5 per cent solutions of the enzyme there were definite ring formations, and after the serum and enzyme were mixed, definite turbidity and precipitate. In solutions of 0.25 and 0.125 per cent there were questionable rings, but after mixing no turbidity nor precipitate could be detected. The sera of two rabbits that had received

TABLE II

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Beef Trypsin

See Table I for explanation of symbols.

Guinea pig No.	Reaction to			
	Beef trypsin	Pig trypsin	Chymotrypsin	Chymotrypsinogen
3	LA 10^{-5} −			
	LB 10^{-4} +			
	RB 10^{-3} +	RA 10^{-3} −		
4	LB 10^{-3} +	LA 10^{-3} −		
	RA 10^{-4} +	R^{w*}B 10^{-2} ?		
5	L^{w}B 10^{-3} ?	LA 10^{-2} ?		
	RB 10^{-3} +		RA 10^{-2} −	
6	LC 10^{-4} +	LA 10^{-3} −		LB 10^{-3} −
	RB 10^{-4} +		RA 10^{-3} −	
7	LB 10^{-4} +		LA 10^{-2} −	
	RB 10^{-4} +	RA 10^{-3} −		
8	LB 10^{-4} +		LA 10^{-3} −	
	RB 10^{-4} +			RA 10^{-2} −
9	LC 10^{-4} +		LA 10^{-2} ?	
			L^{w}B 10^{-3} −	
	RB 10^{-4} +		RA 10^{-3} −	
10	LB 10^{-3} +			LA 10^{-3} −
	RB 10^{-3} +		RA 10^{-3} −	
11	LB 10^{-3} +			LA 10^{-3} −
	RB 10^{-3} +		RA 10^{-3} −	

Three guinea pigs were not sensitive to the trypsin solution.

*w indicates that the horn was washed and allowed to relax before testing.

TABLE III

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Chymotrypsinogen

See Table I for explanation of symbols.

Guinea pig No.	Reaction to		
	Chymotrypsinogen	Chymotrypsin	Beef trypsin
12	LB 10^{-3} +	LA 10^{-3} −	
	RB 10^{-3} ±		RA 10^{-3} −
13	LB 10^{-3} ?	LA 10^{-2} −	
	RA 10^{-3} +		
14	LB 10^{-4} +	LA 10^{-3} −	
	RB 10^{-3} +	RA 10^{-2} −	
15	LA 10^{-5} −		
	LB 10^{-4} +		
	RB 10^{-3} +	RA 10^{-3} −	
16	LB 10^{-3} +	LA 10^{-3} −	
		RA 10^{-2} −	
17	LB 10^{-3} +	LA 10^{-3} −	
	R^{w}B 10^{-3} ±	RA 10^{-2} −	
18	LA 10^{-4} +		
	RD 10^{-4} −	RA 10^{-4} −	
	RE 10^{-3} +	RB 10^{-3} −	
		RC 10^{-2} −	
19	L^{w}B 10^{-3} +	LA 10^{-3} −	

Two guinea pigs were not sensitive and four gave reactions with chymotrypsin as well as chymotrypsinogen.

TABLE IV

Results of Tests for Sensitiveness of Uteri of Guinea Pigs Receiving Chymotrypsin

See Table I for explanation of symbols.

Guinea pig No.	Reaction to	
	Chymotrypsin	Chymotrypsinogen
20	LA 10^{-3} +	
	RC 10^{-2} +	RA 10^{-3} −
		RB 10^{-2} −
21	LA 10^{-3} +	
		RA 10^{-2} −

Two guinea pigs were not sensitive.

repeated injections of chymotrypsinogen failed to cause rings or precipitate when the undiluted serum was mixed with solutions of the enzyme of 1 per cent and higher. None of these sera when injected into guinea pigs sensitized these animals passively to the enzyme. These experiments indicate that precipitins are not easily formed by these enzymes when they are injected into rabbits.

SUMMARY

It has been shown that trypsin from swine and cattle as well as chymotrypsin and chymotrypsinogen, crystallized by Northrop and Kunitz, will act as antigens in that they will actively sensitize guinea pigs. It has been further shown that these four substances can be differentiated, one from the other, by the anaphylactic test. In a limited number of experiments it was shown that precipitins are not readily formed when chymotrypsin and chymotrypsinogen are injected into rabbits.

BIBLIOGRAPHY

1. Kunitz, M., and Northrop, J. H., *Science*, **78**, 558 (1933).
2. Northrop, J. H., *J. Gen. Physiol.*, **13**, 739 (1929–30).
3. Sumner, J. B., and Kirk, J. S., *Science*, **74**, 102 (1931).
4. Kirk, J. S., *J. Biol. Chem.*, **100**, 667 (1933).
5. Kirk, J. S., and Sumner, J. B., *Proc. Soc. Exp. Biol. and Med.*, **29**, 712 (1931–32).
6. Dale, H. H., in Privy Council, Medical Research Council, A system of bacteriology in relation to medicine, London, **9**, 229 (1931).

THE AVAILABILITY OF COPPER IN VARIOUS COMPOUNDS AS A SUPPLEMENT TO IRON IN HEMOGLOBIN FORMATION*

By M. O. SCHULTZE, C. A. ELVEHJEM, and E. B. HART

(*From the Department of Agricultural Chemistry, University of Wisconsin, Madison*)

(Received for publication, June 6, 1934)

The fact that copper is essential to supplement iron in hemoglobin formation is now generally accepted. Evidence which has led to this conclusion is based largely upon experimental work with young anemic rats subsisting on a milk diet. The value of milk as a basal diet for work on anemia is obvious because of its relatively low iron and copper content, because it can be handled with a minimum of contamination, and because experimental animals show good growth on a milk diet supplemented with iron, copper, and manganese.

The amounts of copper necessary to supplement iron in the cure of nutritional anemia are exceedingly small; 0.005 mg. of Cu per day being enough to give a distinct response when fed with sufficient iron to anemic rats. This is particularly striking when it is considered that rats ingest in the milk about the same amount of copper daily, or even more. Criticism of the work in this laboratory (1, 2) has been made on this basis. Recently we have suggested (3) that the copper, as it is present in the milk, "may not be as available as that added in the form of copper sulfate or that entering the milk from copper contamination." This would imply that there are copper compounds which when ingested by the animal are not capable of producing the typical effect of inorganic copper salts in anemic animals having access to available iron. Failure of the copper compounds to effect this

---

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

This investigation was supported in part by a grant from the University Research Fund and the Wisconsin Alumni Research Foundation.

action could be due to the following conditions: (*a*) The copper compound passes through the gastrointestinal tract without being absorbed; (*b*) the copper compound is absorbed but cannot take effect on account of its complexity; (*c*) the copper of the complex cannot be liberated in the animal organism and converted into an effective form.

Newer work on iron metabolism has shown that the hematopoietic value of a food material with respect to iron is not represented by its total iron content since not all iron compounds can be utilized for hemoglobin formation (4). It is well established that hematin iron cannot be utilized effectively by the animal for hemoglobin formation (5, 6). Because plant and animal tissues are known to contain considerable amounts of hematin iron the question of availability of iron in foods for hemoglobin formation is of greatest importance.

It is generally assumed that normal animal and human diets contain sufficient copper to meet the requirements for hemoglobin formation. Analyses of a large number of feeds and food materials have indicated the wide distribution of copper (7, 8). Little, if anything, however, is known about the form in which it is present in animal and plant tissues; whether it exists in the form of salts or in organic combination. To our knowledge, only two naturally occurring organic copper compounds have been described and more or less characterized. Turacin, the reddish violet pigment of the feathers of turacou birds has been identified as a copper uroporphyrin (9). The respiratory pigment of certain Crustacea contains copper. Although the nature of the copper complex of the hemocyanins is not known, it has been definitely proved that hemocyanins do not contain a porphyrin (10). The occurrence of other organic copper compounds, although not yet established, is not unlikely.

Using mature dogs subsisting on a diet low in iron content and made anemic by bleeding or by phenylhydrazine treatment, Handovsky (11) observed the effect of various copper compounds on hemoglobin and erythrocyte regeneration. Fed at a level of 0.5 to 0.6 mg. of copper per kilo of body weight daily, copper as "copper protein" and copper tyrosine appeared to have a marked hematogenic effect, that of copper glycocoll and copper acetate, however, was only slight. The interpretation of these results is rather difficult since no attempt was made to deplete the animals of their stores of copper. Handovsky does not suggest any explanation for the different effect of the various copper compounds.

The present study was initiated to throw some light on the availability of various organic copper compounds as a source of copper necessary for hemoglobin formation.

EXPERIMENTAL

Various organic copper compounds were prepared and fed with an iron supplement to young, severely anemic rats. In the animal experiments the precautions discussed in an earlier paper (3) were observed scrupulously. Hemoglobin determinations were made weekly by the Newcomer method. The copper supplements were fed to the animals daily together with 0.5 mg. of iron added as $FeCl_3$ to milk, which constituted the basal diet. Control animals fed on iron alone and iron plus copper sulfate were carried along with the experimental animals as indicated in Chart I.

In the preparation of the organic copper compounds great care was taken to remove all of the uncombined copper and to prevent further contamination. The success of a study of this nature depends largely on this point.

Copper Caseinate—Casein was prepared from skim milk, washed twice with distilled water, and dissolved in slightly alkaline solution. A 5 per cent solution of $CuSO_4$ was added slowly, with stirring, until precipitation was complete. The solution was neutral to litmus at this point. After filtration and washing the suspended copper caseinate was dialyzed in a collodion bag against distilled and redistilled water for 5 days. The fine suspension of copper caseinate (Cu content 0.1 per cent) was preserved with 0.2 per cent sodium benzoate and fed to the animals in this form. When 5 cc. of the suspension were treated with KCNS and pyridine, and chloroform was added to the mixture, the chloroform layer gave no evidence of the presence of the Cu ion. The supernatant protein suspension, however, assumed a green color.

Glycine Amide Biuret—This compound was prepared from glycine amide by the method of Rising and Yang (12). The glycine amide was made from glycine by ammonolysis of the ethyl ester (13). The product, recrystallized with anhydrous ether three times from absolute alcohol, containing a little NH_4OH (to remove excess copper), was obtained in the form of bluish pink crystals; m.p. 193°, uncorrected.

Analysis (*14*)—$CuC_4H_{12}N_4O_3$. Calculated. Cu 27.91
Found. " 28.03, 28.12

The biuret complex is readily soluble in water. In acid solution it is apparently unstable since the solution becomes decolorized at once upon addition of acid.

Alanine Amide Biuret—This compound was prepared from alanine by the method used for the preparation of glycine amide biuret. The compound was readily obtained in solution. Attempts to isolate it led to much decomposition so that a solution of it, prepared with less than the calculated amount of Cu was used for feeding purposes. A small amount of amorphous solid material which was isolated melted at 223–225° uncorrected. (Rising and Yang (12) report a decomposition point of 227–231°.)

Copper Hematoporphyrin—Hemin was prepared from defibrinated calf blood (15). It was converted into hematoporphyrin by the method of Willstätter and Fischer (16). The copper compound was prepared by dissolving the porphyrin in glacial acetic acid and adding to the boiling solution the calculated amount of copper acetate in boiling glacial acetic acid. Upon cooling, precipitation of the amorphous copper compound began and became complete upon dilution with redistilled water. It was washed several times with redistilled water by decantation, then transferred to a collodion bag, and dialyzed first against dilute acetic acid (1:2000) and then against redistilled water for a total of 5 days. After filtration and drying *in vacuo* the copper hematoporphyrin was obtained in the form of a fine purplish red powder.

Analysis—$C_{34}H_{36}O_6N_4Cu$. Calculated. Cu 9.61
Found. " 10.00, 9.94, 9.74

To test the stability of copper hematoporphyrin under conditions similar to those prevailing in the upper intestine of the animal a digestion experiment was set up after the following plan: 0.5 mg. of Cu as copper hematoporphyrin was incubated at 38° for 70 hours with and without pepsin at a pH of 2. The filtrate was tested with KCNS, pyridine, and $CHCl_3$ for the presence of copper. In no case could any trace of copper be detected. When a known solution of $CuSO_4$ was added to the filtrate, the presence of copper could be detected by a green tinge of the $CHCl_3$ layer after 0.008 mg. of copper had been added. The filtrates contained therefore less than 0.008 mg. of copper.

For feeding purposes copper hematoporphyrin was dissolved in 2 per cent sodium carbonate and fed with iron in the milk.

Hemocyanin—Through the kindness of Dr. A. C. Redfield, Harvard University, we were able to study the availability of the copper in hemocyanin. The blood of *Limulus polyphemus* as it was sent to us by Dr. Redfield was dialyzed against 0.0001 N NaOH (redistilled water was used) for 11 days; then, after the addition of a little $(NH_4)_2SO_4$, it was coagulated by heat, washed with redistilled water, dialyzed against redistilled water for 2 days, filtered dry, washed with redistilled absolute alcohol, and dried. On analysis of the dried product, 1.59 and 1.63 mg. of Cu per gm. were obtained. Redfield *et al.* (17) have reported a copper content of 1.73 mg. of Cu per gm. of hemocyanin.

Whole Wheat—To study the availability of copper in natural foodstuffs such as cereals, the material under investigation may be fed together with milk and adequate amounts of iron to severely anemic rats. In such a procedure the copper of the added food material will serve to supplement the iron in hemoglobin formation. The rate and extent of hemoglobin regeneration of the animals may then be taken as an index of the availability of the copper in the food materials.

In this manner we have fed whole wheat to severely anemic rats at a level that would supply 0.01 mg. of copper daily. Litter mates were fed an equivalent amount of copper sulfate.

DISCUSSION

From Chart I it is evident that the copper in copper caseinate, glycine amide biuret, alanine amide biuret, and hemocyanin can be readily utilized by the anemic rat to supplement iron in the synthesis of hemoglobin. The level of copper required in the form of the compounds mentioned is apparently not higher than that of copper sulfate. Considering the stability towards dilute acid, one should expect the copper of copper caseinate, glycine amide biuret, and alanine amide biuret to be available to the animal since the colored copper complexes in all three cases become colorless in acid solution and give positive tests for inorganic copper. These copper compounds probably yield their copper in the acidic portions of the alimentary canal of the rat.

Barkan (18) has shown that the copper of hemocyanin becomes dialyzable after incubation with dilute acid. Digestion experiments, which we carried out, indicated that upon incubation of hemocyanin in acid and in acid-pepsin solution the copper can be largely recovered in the filtrate. These observations are in agreement with the results of our animal experiments. Copper in the form of hemocyanin was just as efficient (at the levels fed) to supplement iron for hemoglobin regeneration as was copper sulfate.

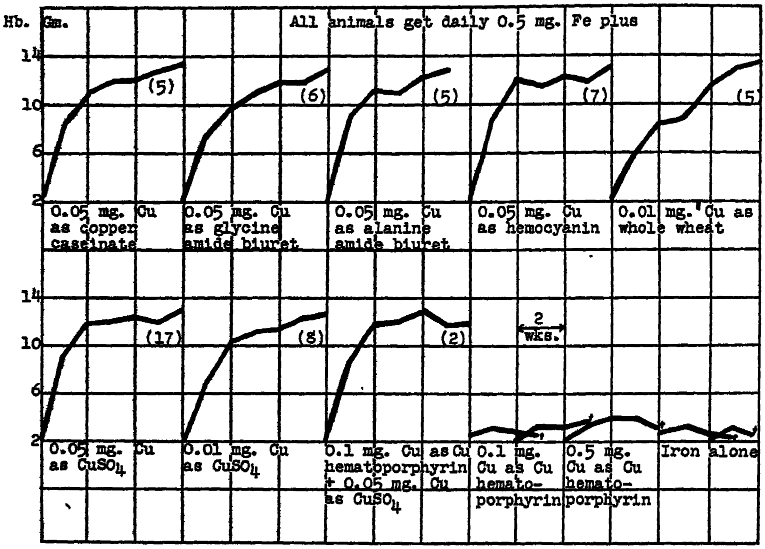


Chart I. Availability of copper for hemoglobin formation. Composite curves of hemoglobin regeneration of rats receiving copper from various compounds are given. The number of animals used in each group is indicated in parentheses. Representative response curves are given for rats receiving copper hematoporphyrin (12) and iron only (15) because differences in period of survival do not permit composite curves.

In striking contrast to the effect of the copper compounds discussed is that of copper hematoporphyrin. Of the twelve rats that were fed this compound as a source of copper nine died without showing any appreciable hemoglobin regeneration. Two rats getting daily 0.25 and 0.50 mg. of copper respectively as copper hematoporphyrin for 11 weeks had hemoglobin concentrations of 6.80 and 8.50 gm. per 100 cc. at the end of this period. They died shortly afterwards. The presence of traces of ionic copper in the copper hematoporphyrin might account for such a slow hemoglobin regeneration. No toxic effects of the copper hematoporphyrin could be observed. Two rats getting 0.05 mg. of copper sulfate in addition to the copper hematoporphyrin and iron showed normal recovery from anemia.

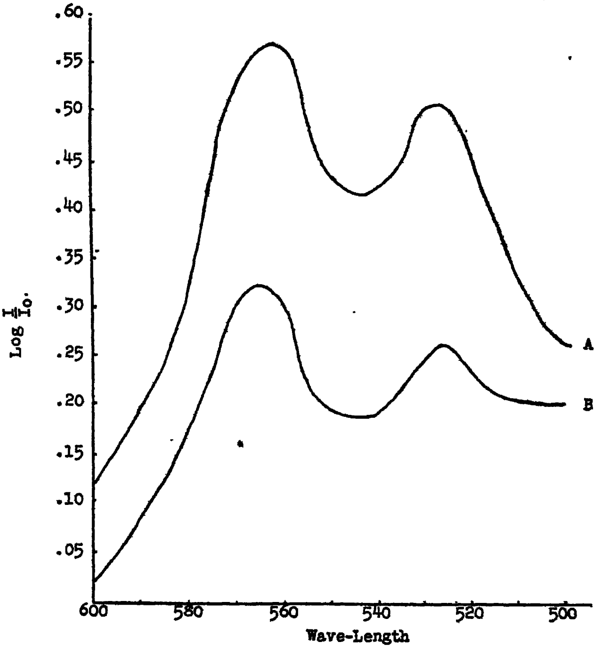


Chart II. Curve A, absorption spectrum of Cu hematoporphyrin; Curve B, absorption spectrum of fecal extract from rat fed Cu hematoporphyrin.

Chart II represents the absorption spectra of a solution of copper hematoporphyrin in 2 per cent Na_2CO_3 (diluted 1:64) and of a 2 per cent Na_2CO_3 extract (diluted 1:4) of feces of rats getting 0.5 mg. of Cu as copper hematoporphyrin (5.1 mg.) daily. In the latter case the light absorption of the fecal extract was compared with that of a 2 per cent Na_2CO_3 extract of feces of rats subsisting on a milk-iron diet. In this manner the effect of fecal matter on light absorption could be reduced. The curves obtained with a Bausch and Lomb Universal spectrophotometer show maximal absorption at the same wave-lengths. This indicates that the feces of rats getting copper hematoporphyrin contained considerable amounts of this compound. In view of the insolubility of the copper hematoporphyrin in acid solution, this is not surprising. It probably explains why the animals were unable to utilize the copper administered in the form of a porphyrin complex.

Application of the biological method to a study of the availability of copper in natural food materials entails some difficulties. It is possible that not all of the copper in the foodstuff is present is the same form and may therefore not have the same availability. To detect this biologically it is necessary to feed the supplement with sufficient iron at such a level that its total copper content, if wholly available, will permit a fairly rapid hemoglobin regeneration. From our experiences with feeding copper to rats at different levels a daily dosage of 0.01 mg. of copper appears to be most desirable. With copper sulfate fed at this level recovery is quite rapid although not maximal. If more than 0.01 mg. of total copper were fed in the form of natural food materials partial availability might be obliterated by sufficient available copper. At lower levels of total copper individual variations in response of the anemic animals would complicate the interpretation of recovery curves. Furthermore it must be borne in mind that the addition of natural food materials to the milk-iron diet may improve the severe condition of the anemic rats through factors other than their content of available copper. The animals receiving daily 0.01 mg. of total copper in the form of food materials added to the milk-iron diet are therefore not strictly comparable to the control animals getting 0.01 mg. of available copper as copper sulfate.

Chart I includes the curve of hemoglobin regeneration of rats getting 0.01 mg. of copper daily in the form of whole wheat and a similar curve of control rats getting 0.01 mg. of copper as copper sulfate. It is evident that the copper in whole wheat is readily available to supplement iron in hemoglobin formation.

Further work will have to show whether the copper of most of the naturally occurring foods is readily available or whether it may partly exist in a form that is not assimilated by the animal. That this is possible has been demonstrated in this study.

These considerations must be borne in mind in future studies on the value of food materials for hemoglobin regeneration. The results of the work of Rose and associates (19) are undoubtedly complicated by the fact that although the total daily copper intake of the rats was sufficient for good hemoglobin regeneration, much of this copper was contributed by the ingested milk and may therefore not have been completely available for hemoglobin formation. Recent observations by Bing and co-workers (20) that the copper of a milk-iron diet is assimilated to only a small extent might be also explained on this basis.

SUMMARY

1. The copper of copper caseinate, glycine amide biuret, alanine amide biuret, hemocyanin from *Limulus polyphemus*, and of whole wheat is readily utilized by severely anemic rats to supplement iron for hemoglobin formation.

2. Under the same conditions copper hematoporphyrin is not utilized even when fed at high levels. It passes through the alimentary tract unchanged.

3. The question of availability of copper has been discussed.

BIBLIOGRAPHY

1. Lintzel, W., *Ergebn. Physiol.*, **31**, 893 (1933).
2. Beard, H. H., and Myers, V. C., *J. Biol. Chem.*, **94**, 71 (1931-32).
3. Schultze, M. O., and Elvehjem, C. A., *J. Biol. Chem.*, **102**, 357 (1933).
4. Elvehjem, C. A., Hart, E. B., and Sherman, W. C., *J. Biol. Chem.*, **103**, 61 (1933).
5. Lintzel, W., *Z. Biol.*, **87**, 97 (1928).
6. Elvehjem, C. A., *J. Am. Med. Assn.*, **98**, 1047 (1932).
7. Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **82**, 473 (1929).
8. Lindow, C. W., Elvehjem, C. A., and Peterson, W. H., *J. Biol. Chem.* **82**, 465 (1929).
9. Fischer, H., and Hilger, Y., *Z. physiol. Chem.*, **138**, 49 (1924).
10. Schmitz, A., *Z. physiol. Chem.*, **194**, 232 (1931).

11. Handovsky, H., *Klin. Woch.*, **11**, 981 (1932). Handovsky, H., and von Cotzhausen, D., *Arch. exp. Path. u. Pharmakol.*, **173**, 187 (1933).
12. Rising, M. M., and Yang, P. S., *J. Biol. Chem.*, **99**, 755 (1932–33).
13. Yang, P. S., and Rising, M. M., *J. Am. Chem. Soc.*, **53**, 3183 (1931).
14. Elvehjem, C. A., and Lindow, C. W., *J. Biol. Chem.*, **81**, 435 (1929).
15. Nencki, M., and Zalesky, J., *Z. physiol. Chem.*, **30**, 384 (1900).
16. Willstätter, R., and Fischer, M., *Z. physiol. Chem.*, **87**, 459 (1913).
17. Redfield, A. C., Coolidge, T., and Shotts, M. A., *J. Biol. Chem.*, **76**, 185 (1928).
18. Barkan, G., *Klin. Woch.*, **12**, 546 (1933).
19. Rose, M. S., Vahlteich, E. M., and MacLeod, G., *J. Biol. Chem.*, **104**, 217 (1934).
20. Bing, F. C., Saurwein, E. M., and Myers, V. C., *J. Biol. Chem.*, **105**, 343 (1934).

A MICROMETHOD FOR THE DETERMINATION OF FREE AND COMBINED CHOLESTEROL*

By RUDOLF SCHOENHEIMER AND WARREN M. SPERRY

(*From the Department of Biological Chemistry, College of Physicians and Surgeons, Columbia University, and the Chemical Laboratory, Babies Hospital, New York*)

(Received for publication, April 7, 1934)

In this communication we present an accurate, rapid, and relatively simple procedure for the determination of free and total cholesterol in blood or other biological material. The method has been especially designed for serial analyses, and a single worker can carry out sixteen to eighteen analyses per day with ease. Only 0.2 cc. of serum or whole blood is required for the determination of both free and combined cholesterol, and it is possible, therefore, to utilize finger blood for the analysis. Amounts of cholesterol ranging between 0.02 and 0.15 mg. may be determined.

In principle our procedure consists in the precipitation of cholesterol with digitonin followed by the application of a color reaction to the precipitate. The isolation of cholesterol as digitonide entirely avoids the errors which are introduced into other colorimetric methods by other chromogenic substances present in fatty extracts. Conditions have been discovered under which cholesterol digitonide is completely insoluble and may be readily isolated by centrifugation.[1]

Yoshimatsu has described (2) a method based on the color

---

* This investigation was made possible by the assistance of the Josiah Macy, Jr., Foundation.

This investigation was carried out in part in the Chemical Department, Institute of Pathology, University of Freiburg, Germany.

[1] See Schoenheimer and Dam (1) for a discussion of the precipitation of cholesterol and the solubility of cholesterol digitonide. The development of our method has occupied over 2 years in the Freiburg and New York Laboratories. The principal difficulty has been to find conditions for the complete precipitation of small amounts of cholesterol.

developed by cholesterol digitonide with the phenol reagent of Folin and Denis. The digitonide is precipitated in 95 per cent alcohol; 96 per cent alcohol has been shown (1) to dissolve no less than 0.02 per cent of digitonide corresponding to 0.1 mg. of cholesterol in the 2 cc. of alcohol employed by Yoshimatsu. Since less than 0.1 mg. is to be expected from amounts of blood analyzed by Yoshimatsu's procedure, the error due to solubility must be considerable despite the large excess of digitonin which he employs.

While this investigation was in progress, Obermer and Milton published a method (3) in which a color reaction (Bernoulli) is also applied to the digitonide of cholesterol. In our hands the Bernoulli reaction has yielded highly inconsistent results with known samples of pure cholesterol. Furthermore, as is pointed out by Obermer and Milton, digitonin gives considerable color which we have found to have an absorption curve similar to that of the color from cholesterol. With this procedure it would, therefore, be necessary to wash out excess digitonin quantitatively.

The very weak color produced by digitonin with the modified Liebermann-Burchard reaction, which we employ, has no absorption over the range in which the color developed by cholesterol exhibits its maximum absorption (610 to 620 $\mu\mu$). Therefore, by using a color filter which transmits light only over this range, it is possible to compare the color given by digitonide directly with that of cholesterol alone in any suitable apparatus. It is not necessary to wash out excess digitonin quantitatively; this operation, essential in all gravimetric and oxidative cholesterol methods, would, on a micro scale, involve considerable danger of dissolving appreciable amounts of digitonide.

With control of temperature and reaction time the color developed with pure cholesterol digitonide is reproducible within the limit of error of the instrument used for measuring color. It is possible, by using a sensitive photometer, to achieve a degree of accuracy which has hitherto been obtainable only with the macrogravimetric procedure of Windaus, which requires 15 to 20 cc. of blood or serum. We have employed the Zeiss Pulfrich photometer, equipped with 50 mm. microcells, almost exclusively. Any ordinary colorimeter may be used if equipped with a suitable color filter of narrow transmission range, and with microcells approximately 50 mm. in depth and containing not over 2 cc. However, it must be remembered that the accuracy possible with such an apparatus is considerably less than with a sensitive photometer.[2]

The time required for the analysis is relatively short. Only a few minutes are needed to make an extract, and since such small amounts of material are used, it is not necessary to concentrate the solution as is the case in most cholesterol methods.

Procedure

Reagents

1. Acetone-absolute alcohol (1:1).
2. Ether.[3]
3. Acetone-ether (1:2).
4. Digitonin solution. This solution is prepared by dissolving 1 gm. of digitonin in 1 liter of distilled water. The solution is placed in the ice box for at least 24 hours (preferably longer) and then centrifuged to pack the precipitate[4] which forms. The supernatant solution is filtered and concentrated to approximately 500 cc. This is best done by placing the solution in a weighed 1 liter flask equipped with inlet and outlet tubes. The mouth of the inlet tube should be kept about 2 cm. above the surface of the solution. A rapid stream of air, filtered through cotton, is blown, or drawn by suction, through the flask, which is immersed in boiling water. The point at which the required amount of water has been removed is determined with sufficient accuracy by weighing the flask with its contents on an ordinary laboratory balance. The concentration requires 3 to 4 hours. Should a sediment appear in

---

[2] See foot-note 20 cited in the discussion of "Accuracy."

[3] All the ether used in our laboratory is prepared routinely by washing with a sodium sulfite solution followed by several washings with water. The washed ether is distilled over calcium chloride. We have no data on the use of unwashed ether in the determination of cholesterol.

[4] The nature of this substance, which precipitates only from dilute aqueous solutions, is not known. It forms rapidly in a 0.1 per cent solution but comes down slowly in a 0.2 per cent solution. We have some evidence indicating that it tends to give high values when left in the solution though we have not studied the point extensively. We have employed only Hoffmann-La Roche, Inc., digitonin which we have found to give uniform results. It is possible, in view of the findings of Schoenheimer and Dam (1), that other samples of digitonin may not behave similarly.

the concentrated solution on standing, it should be removed by filtering.

5. Potassium hydroxide solution made by dissolving 10 gm. of pure KOH in 20 cc. of water.

6. Hydrochloric acid, approximately 5 per cent, made by diluting 15 cc. of concentrated HCl to 100 cc.

7. Acetic acid, 100 per cent. We have found the Eastman and Kahlbaum products to be equally good.

8. Acetic anhydride. The product, labeled "acetic anhydride 99–100 per cent," supplied by the Eastman Kodak Company has been uniformly satisfactory.[5]

9. Concentrated sulfuric acid.

Special Apparatus Required

1. Volumetric flasks, 5 cc.
2. Funnels, 2.5 cm. in diameter.
3. Filter paper, 4.5 cm. in diameter. The filter paper must be extracted with ether or hot alcohol until completely free of sterols.
4. Stirring rods, approximately 13 cm. long.
5. Preserving jars, either pint or quart sizes, with rubber gaskets.
6. Dropping bottles with ground-in pipettes equipped with rubber bulbs.
7. Glass tube, 6 to 7 mm. in diameter, drawn out to a fine capillary. The capillary tip should be about 8 cm. long.
8. Transfer pipettes, for use with rubber bulbs. These pipettes are made by drawing out 8 mm. glass tubing. The total length should be approximately 13 cm., and the tip should be approximately 5 cm. long. The orifice should be large enough to permit rapid filling and emptying.
9. Water bath, equipped to hold 15 cc. centrifuge tubes in the dark at 25°.[6]
10. Zeiss Pulfrich photometer equipped with 5 cm. microcells, or any other color-measuring instrument, of equivalent accuracy,

---

[5] Kahlbaum's acetic anhydride "pro analysis" gives values approximately 5 per cent lower than the Eastman preparation. A fraction, boiling at 137° (about two-thirds of the original sample), obtained by fractionation of the Kahlbaum acetic anhydride with an efficient column, gave the same values as the Eastman product.

[6] Any pan of fairly large capacity, fitted with a rack and a thermometer, is suitable. The pan may be placed in a box with a door or simply covered with a dark cloth to exclude light.

in which cells at least 5 cm. long and containing not over 2 cc. may be used.

Extraction of Serum or Whole Blood—Approximately 3 cc. of the acetone-absolute alcohol solution are placed in a 5 cc. volumetric flask and brought to a boil on the steam bath; 0.2 cc. of serum or whole blood is added slowly to the hot solution from a capillary pipette with shaking.[7] The stopper is inserted and the flask is shaken vigorously to break up any clumps of protein which may have formed. The stopper is removed, washed, and the contents of the flask are again heated to boiling. The flask is cooled to room temperature,[8] and the contents are made up to volume with alcohol-acetone, mixed thoroughly, and filtered through a small, dry filter. The filtrate should be perfectly clear. In case duplicate determinations are desired, the amounts may be increased in approximate proportion. Good results have been obtained with extracts made up in 10 cc. flasks from 0.5 cc. of serum.

Precipitation of Free Cholesterol[9]—2 cc. of the filtrate are pipetted into an ordinary 15 cc. conical centrifuge tube, 1 cc. of the digitonin solution is added, and the solution is stirred thoroughly with a stirring rod which is left in the tube. The tube is placed in a preserving jar (pint or quart size), the cover is placed on tightly, and the jar is left overnight[10] at room temperature. After standing the tube is removed to a test-tube rack and the solution is stirred gently to free particles of precipitate which may adhere to the walls of the tube; the stirring rod is removed carefully without touching the upper part of the tube and laid out on a rack so designed that no adherent precipitate is rubbed off;[11] and the tube is centrifuged 15 minutes at about 2500 R.P.M. The supernatant solution is drawn off slowly with the fine capillary pipette, to which suction is cautiously applied from a pump.[12] Care is taken not to touch the wall of the centrifuge tube with the pipette nor to stir up the precipitate. A few particles, which are probably cholesterol ester, usually float at or near the surface and are drawn off with the solution.

---

[7] In case the cholesterol is expected to be high (350 mg. or more per 100 cc.), the ratio of serum taken to volume of extract should be reduced.

[8] The accuracy may be increased somewhat by working always at the same temperature as the coefficient of expansion of acetone-alcohol is relatively high.

[9] The procedure is described for a single determination. In practise the average time per determination may be greatly shortened by carrying through a number of analyses (preferably sixteen to eighteen) together.

[10] Results of sufficient accuracy for ordinary purposes may be obtained with 1 hour's standing (see discussion, p. 754).

[11] We use a rack made of heavy wire designed to hold a number of stirring rods. When several samples are run together (as is usually the case), the position of the rods is numbered so they may be replaced in the proper centrifuge tubes.

The stirring rod is placed in the tube, and the wall of the tube and the rod are washed down with 1.5 to 2.0 cc. of the acetone-ether solution. The wash solution is best added from a dropping pipette with a rubber bulb attached. The precipitate is stirred up thoroughly, the rod is removed to the rack, the tube is centrifuged for 5 minutes, and the supernatant solution is removed as before. The suction must be reduced somewhat as the acetone-ether solution is drawn off more rapidly than the more viscous water-alcohol-acetone solution. The precipitate is washed twice more just as before, except that ether instead of acetone-ether is used. After the last wash solution is removed, the stirring rod is placed in the tube, which is then placed in a water bath at about 40°. The precipitate becomes dry in 2 or 3 minutes. The last traces of ether may be removed by holding a tube, attached to a suction pump, in the test-tube for a few moments. The sample is now ready for color development.

Precipitation of Total Cholesterol—1 cc. of the extract is pipetted into a 15 cc. centrifuge tube and 1 drop of the KOH solution is added[13] and stirred into solution with a stirring rod which is left in the tube. The tube is placed in a preserving jar containing a layer of sand about 3 cm. deep which has been heated to about 40°. The sand acts as a heat reservoir. The cover is clamped on tightly and the jar is kept at 37–40° (we utilize an ordinary incubator) for ½ hour.

---

[12] The suction is reduced if necessary by admitting air through a side tube.

[13] The size of the drop may be varied within wide limits by changing the height of the burette, or pipette tip above the surface of the solution. The closer the tip, the smaller will the drop be as acetone and alcohol vapor lower the surface tension of the alkali solution. A few trials with blank samples will determine the proper position of the tip. The drop should be of such a size that approximately 0.1 cc. of 5 per cent HCl solution are required to neutralize it.

After hydrolysis the tube is cooled, 1 cc. of the acetone-alcohol solution is added, and the solution is titrated with 5 per cent HCl (phenolphthalein indicator). The solution is stirred after the addition of each drop, and care is taken to insure that a definite excess of acid is present. Small amounts of HCl have no effect on the precipitation of cholesterol with digitonin, but alkali interferes. 1 cc. of digitonin solution is added, the solution is stirred thoroughly (the suspended salt dissolves immediately),[14] the tube is allowed to stand 1 hour or longer, and the precipitate is centrifuged 5 minutes and washed just as has been described for free cholesterol except that only one ether washing is necessary.

Development and Reading of Color—The dried precipitate of cholesterol digitonide is now dissolved in 1 cc.[15] of acetic acid. Care is taken, when adding the acid, to wash down the wall of the centrifuge tube in case any particles of digitonide may have adhered to it. Solution may be hastened by warming the tube in a water bath at about 60° and stirring.

Next, the temperature of the water bath is adjusted to 25°, and the tubes[16] are placed in the bath and left for a few moments to bring them to temperature equilibrium. One is removed, placed in a rack in a small pan, or beaker, containing water at 25°. 2 cc. of acetic anhydride are added, followed by 0.1 cc. of concentrated H_2SO_4, best added from an automatic microburette.[17] The solution is now stirred vigorously (the original stirring rod is still in the tube), and the tube is replaced in the water bath. Another tube is removed to the small bath and the reagents added as before. The interval between the addition of reagents is so timed that not less than 27 minutes nor more than 37 minutes elapse between the addition of H_2SO_4 and reading. With practise about 2 minutes are required for a reading; it is possible, therefore, to carry through sixteen to eighteen determinations in a series. The temperature of the bath is kept at 25° during the procedure.

---

[14] Care must be taken to dissolve any salt adhering to the wall of the tube above the surface of the solution.

[15] In case the precipitate appears to be too large to be read, the analysis may be saved by adding double amounts of acetic acid, acetic anhydride, and H_2SO_4. When this step is indicated, can only be learned by experience.

[16] The procedure of color development and reading is described for a series of determinations (see foot-note 9).

[17] The measurement of 0.1 cc. of H_2SO_4 is difficult since drainage is slow. We have found it best to measure the acid by counting drops. 4 drops from our burette are equivalent to almost exactly 0.1 cc.

In reading the color with the Pulfrich photometer one of the cells is filled with a blank solution (1 cc. of acetic acid, 2 cc. of acetic anhydride, and 0.1 cc. of H_2SO_4); the other cell is filled with the unknown colored solution, a transfer pipette being used.[18] The percentage transmission is read, the cells are reversed, and the reading is taken on the opposite side of the instrument (see manufacturer's directions for details). The solution is poured out, and the cell is washed out twice with approximately 0.5 cc. portions of the next unknown solution, a clean transfer pipette being used. The percentage transmission is read as before.

TABLE I

Specific Extinction Coefficients of Different Amounts of Cholesterol Precipitated According to Procedure Described

Amount	No. of determinations	Average $E_{sp.}$	Standard deviation	Probable error*
mg.				
0.15	15	1.447	±0.0111	±0.0019
0.10	18	1.454	±0.0110	±0.0017
0.05	20	1.449	±0.0226	±0.0034
0.04	11	1.454	±0.0115	±0.0023
0.03	12	1.447	±0.0226	±0.0044
Average..................		1.450	±0.0174	±0.0013

* With 0.025 mg. the probable error was found to be somewhat larger than the values shown. Very little color is produced by such small amounts, and the error in reading is correspondingly great. Amounts smaller than 0.03 mg. are rarely met with in working with serum or blood.

In case an ordinary colorimeter is used, the unknown is compared with a standard solution of anhydrous cholesterol in acetic acid. Since the color fades slowly after reaching the maximum (see discussion, p. 757), it is essential that the time of color development be approximately the same for unknown and standard. This may necessitate the use of more than one standard during a series of readings. Only one *concentration* of standard is necessary, since Beer's law is obeyed.

---

[18] Occasionally an insoluble sediment of unknown nature is present. It settles during the development of color and does not interfere with the reading if care is taken not to stir it up when withdrawing solution for filling the cell.

The calculation of the amount of cholesterol is based on the results of a long series of determinations on known amounts of cholesterol (Table I), in which it was shown that the specific extinction coefficient ($E_{sp.}$) is constant over the range studied (0.03 to 0.15 mg.). $E_{sp.} = E/(\text{No. of mg. of cholesterol})$; $E = (-\log (T/100))/L$ (where T is the percentage transmission and L the length of the cell in cm.). The value of $E_{sp.}$, which should be determined independently by the operator (with pure anhydrous cholesterol), was in this laboratory found to be 1.450. The amount of cholesterol is given by the value, $E_{obs.}/E_{sp.}$[19]

DISCUSSION

In developing this method a great many different modifications of technique have been tested with several thousand known and unknown samples of free and combined cholesterol. Space does not permit a detailed discussion of our experiments. We shall take up only those points which are of especial interest.

Completeness of Precipitation—That the precipitation is complete under the conditions described is shown by the following evidence: (*a*) When different amounts of cholesterol (from 0.025 to 0.15 mg.), precipitated according to our procedure, are plotted against average extinction coefficients, the points fall almost exactly on a straight line which passes through the origin. (*b*) Solutions of pure cholesterol in acetic acid gave the same specific extinction coefficients as cholesterol precipitated according to our procedure (Table II). (*c*) Solutions of cholesterol digitonide in acetic acid gave the same extinction coefficients (calculated from the cholesterol content) within the limit of error (Table II). (*d*) Amounts of pure cholesterol 10 times as great (0.25 to 1.5 mg.) showed essentially the same specific extinction coefficients either when precipitated by a modified form of the microprocedure or when dissolved in acetic acid and determined directly (Table II).

Absorption Curves of Color Developed by Cholesterol and by Cholesterol Digitonide—The color produced by cholesterol digitonide has

[19] When a great many determinations are to be made, it is worth while to prepare a chart on semilogarithmic paper by means of which amounts may be read off directly from photometer readings.

the same absorption as the color produced by pure cholesterol in the portion of the spectrum where cholesterol shows its maximum absorption. This is shown clearly in Chart I. The color was developed in acetic acid solutions of cholesterol and cholesterol digitonide containing the same amount (1 mg. per cc.) of cholesterol. The absorption was measured with a Hilger spectrophotometer.

TABLE II

Specific Extinction Coefficients

Sample	Amount of cholesterol	No. of determinations	Average $E_{sp.}$
	mg.		
Cholesterol in acetic acid..................	0.025	5	1.460
“ “ “ “	0.050	8	1.454
“ “ “ “	0.060	6	1.458
“ “ “ “	0.100	9	1.452
“ “ “ “	0.150	3	1.446
“ “ “ “	0.25*	3	1.476
“ “ “ “	0.40*	2	1.446
“ “ “ “	0.50*	4	1.455
“ “ “ “	0.60*	4	1.451
“ “ “ “	1.00*	6	1.443
“ digitonide in acetic acid........	†	15	1.467
“ pptd. by modified technique....	0.25*	4	1.453
“ “ “ “ “	0.50*	4	1.451
“ “ “ “ “	1.00*	4	1.447
“ “ “ “ “	1.50*	2	1.438

* Read in 0.5 cm. cells.

† Seven different amounts, ranging from 0.023 to 0.1147 mg. (cholesterol content), were measured.

Time Required for Precipitation—In most of our work we have allowed the samples to precipitate approximately 16 hours (overnight). However, we have shown by numerous experiments that the precipitation of standard solutions of free cholesterol or of hydrolyzed cholesterol ester is complete in 1 hour. Unfortunately the precipitation of free cholesterol from blood extracts may occasionally be somewhat slower, probably owing to the presence of other fatty substances in the extract. Values 5 to 10 per cent low (as compared with values obtained with overnight standing) have been found in some cases even after 2 and 3 hours standing. To be sure of accurate results it is necessary to let the samples stand overnight, although fairly good values will be obtained in most cases after 1 hour. In determinations of total cholesterol in blood complete precipitation has always been obtained in 1 hour (see Table III).

Saponification of Cholesterol Esters—We have analyzed a large number of samples of cholesterol oleate with saponification for ½ hour at 37–40°. The values have always been as high as those obtained with free cholesterol under the same conditions of precipitation and reading. A series of twenty-five samples varying from 0.05 to 0.15 mg. of cholesterol equivalent, analyzed according to the final procedure, gave an average specific extinction coefficient of 1.451 ± 0.0028 as compared with an average of 1.450 obtained with free cholesterol.

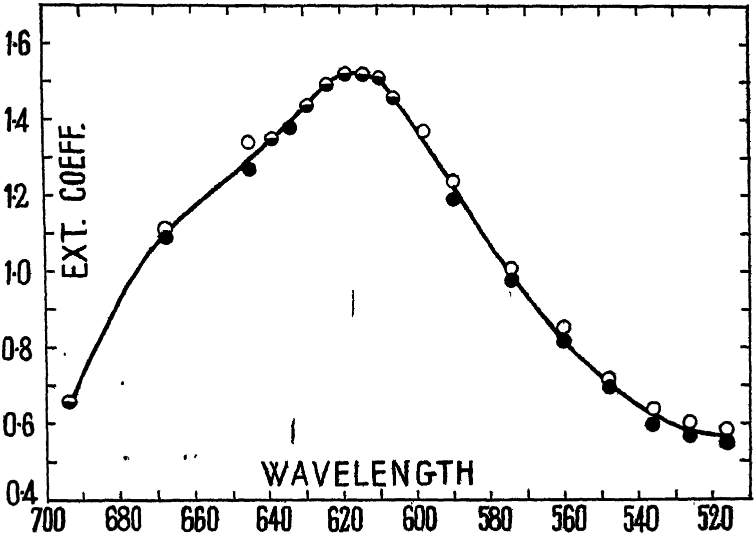


CHART I. Absorption curve for color developed by cholesterol (filled in circles) and cholesterol digitonide (clear circles).

We have relied more on experiments with blood extracts than on determinations of known solutions of cholesterol esters to show that complete saponification is obtained, because the nature of

TABLE III

Determinations of Total Cholesterol in Serum Extracts by Different Procedures

All extracts were made up 1 cc. to 25 cc. with the exception noted (Sm, 0.5 cc. to 25 cc.).

Patient	Procedure*	Alkali†	HCl‡	Time of hydrolysis	Time of precipitation	Total cholesterol
			cc.	*min.*		*mg. per 100 cc.*
Sh.	Indirect	1:1		30	Overnight	184
"	Direct	1:1	0.15	30	1 hr.	184
"	"	1:2	0.11	30	1 "	183
"	"	1:3	0.08	30	1 "	183
Dp.	Indirect	1:1		30	Overnight	250
"	Direct	1:1	0.15	30	1 hr.	250
"	"	1:2	0.11	30	1 "	250
"	"	1:3	0.07	30	1 "	250
Sm.	Indirect	1:1		30	Overnight	858§
"	Direct	1:1	0.15	30	1 hr.	862§
"	"	1:2	0.12	30	1 "	864§
"	"	1:3	0.09	30	1 "	862§
" (0.5 cc. per 25 cc.)	Indirect	1:1		30	Overnight	862
" 0.5 " " 25 "	Direct	1:1	0.14	30	1 hr.	858
" 0.5 " " 25 "	"	1:2	0.11	30	1 "	851
" 0.5 " " 25 "	"	1:3	0.09	30	1 "	862
" (whole blood)	Indirect	1:1		30	Overnight	541
" " "	Direct	1:1	0.12	30	1 hr.	541
" " "	"	1:2	0.10	30	1 "	544
" " "	"	1:3	0.07	30	1 "	536
Ps.	"	1:2		30	1 "	247
"	"	1:2		60	1 "	248
"	"	1:2		90	1 "	247
Cs.	"	1:2		30	1 "	320
"	"	1:2		60	1 "	321
"	"	1:2		90	1 "	321

---

* In the indirect procedure an alcoholic solution of HCl was used for titration and the salt was filtered off before precipitation.

† 1 drop was added in each case. Values indicate the proportion of KOH to water in the solutions used.

‡ Amount of 5 per cent HCl required to neutralize the alkali.

§ Double amounts of reagents were added for development of color so the solutions might be dilute enough to be read.

the esters existing in blood is not known with certainty. Table III presents the results of some of our experiments with serum (and one whole blood) filtrates. 1 cc. portions of the same filtrate were saponified with 1 drop of different solutions of alkali ranging in concentration from 1:1 to 1:3. The time of saponification was varied from 30 to 90 minutes. The same values were obtained in all cases. Even in one filtrate (patient Sm, 1 cc. to 25 cc.) in which very large amounts of cholesterol ester were present (611 mg. per 100 cc. of serum) the saponification was complete in all determinations. The same values were obtained as with a filtrate made up half as concentrated from the same serum.

That the saponification is complete is further demonstrated by comparison with the Windaus macrogravimetric procedure (see Table V).

Precipitation in Presence of Salt—In all of our earlier work the salt precipitate, formed in titration of alkali, was filtered off (with a Jena fritted glass filter) before precipitation. To decrease the solubility of the salt, a solution of HCl in absolute alcohol was used for titration. We have found recently that if the salt is left in the tube, it dissolves in the water added as digitonin solution, and does not interfere at all with the precipitation. This has been shown in experiments with standard solutions of free cholesterol to which alkali was added and titrated as in the determination of total cholesterol, and by experiments with known solutions of cholesterol esters. It is shown conclusively in Table III. The same results were obtained on blood filtrates (the final test of a method) whether the indirect procedure (with filtration) or the direct procedure (with precipitation of digitonides in the presence of salt) was employed.

Development of Color (Effect of Time, Temperature, and Amount of H_2SO_4)—The color formed by the action of acetic anhydride and H_2SO_4 on cholesterol develops to a maximum and then fades slowly. In 1 or 2 hours the original green color changes to a yellow-brown. We have carried out a large series of experiments with acetic acid solutions of cholesterol digitonide (containing 0.1, 0.06, and 0.02 mg. of cholesterol per cc.) to show the effect of temperature and amount of H_2SO_4 on the time of development and fading of the color. In general the results show that the higher the temperature and the larger the amount of H_2SO_4, the earlier the color reaches a maximum and the earlier it fades, but the maximum is the same under all conditions studied. At 25° with 0.1 cc. of H_2SO_4 the maximum is reached at about 27 minutes after the addition of H_2SO_4; fading starts at about 37 minutes. At the same temperature with 0.075 cc. of H_2SO_4 the maximum is not reached until after about 35 minutes, and fading becomes evident at about 45 minutes. When the color is developed at 30°, fading sets in between 25 and 30 minutes after the addition of H_2SO_4. As at 25° fading starts earlier when larger amounts of H_2SO_4 are used.

Accuracy—The accuracy of the method as applied to solutions of pure cholesterol[20] is shown in Table I. The accuracy when applied to blood serum has been tested by duplicate, triplicate, and (in a few cases) quadruplicate analyses of forty-six serums. In making these determinations the proportion of serum to volume of extract was varied widely, and in many cases extracts of 0.2 cc. of serum made up to 5 cc. were compared with extracts of 1 cc. of serum made up to 25 cc. Errors due to incomplete extraction, inaccurate pipetting of small amounts, incomplete precipitation of digitonide, and incomplete saponification of esters should have become evident under these circumstances. The standard deviations of the percentage variations from individual averages are 1.48 per cent for free cholesterol and 1.16 per cent for total cholesterol. A few of the data are presented in Table IV.

Cholesterol added to the alcohol-acetone solution used for extracting sera was recovered within the limit of accuracy of the method (minimum 97.2 per cent, maximum 99.3 per cent recovery).

As the final test of accuracy we have compared the values obtained with our procedure with those found with the Windaus macrogravimetric method (Table V). The error of the Windaus procedure is generally believed to lie within the limits of ±2 per cent, but occasionally the error may be somewhat higher (1). The values found with our method were very close to those obtained with the Windaus procedure.

---

[20] The accuracy possible with an ordinary colorimeter equipped with microcells is indicated by the results of a series of thirty-three determinations of known amounts of cholesterol varying from 0.025 to 0.15 mg. The standard deviation of the percentage errors was 5.0 per cent (maximum deviation, obtained with a sample of 0.025 mg. of cholesterol, 12 per cent). A Wratten color filter, No. 71a (made by the Eastman Kodak Company), was placed over the eyepiece of the colorimeter. We are indebted to the Bausch and Lomb Optical Company for the microcells used in these determinations.

TABLE IV

Determination of Free and Combined Cholesterol in Human Blood Sera

Serum No.	Volume of serum	Volume of extract	Cholesterol in 100 cc. serum			Serum No.	Volume of serum	Volume of extract	Cholesterol in 100 cc. serum		
			Free	Total	Combined				Free	Total	Combined
	cc.	*cc.*	*mg.*	*mg.*	*mg.*		*cc.*	*cc.*	*mg.*	*mg.*	*mg.*
1	0.2	5	43.1	148.0	104.9	6	1.0	25	36.9	108.2	71.3
1	0.3	5	43.7	150.0	106.3	12	0.2	5	48.0	165.0	117.0
1	0.5	10	44.6	147.0	102.4	12	0.3	5	49.0	164.4	115.4
1	1.0	25	43.0	150.0	107.0	12	1.0	25	48.4	168.7	120.3
5	0.2	5	49.5	146.5	97.0	47	0.2	5	99.2	349.0	249.8
5	0.3	5	47.0	146.8	99.8	47	0.2	10	102.5	347.0	244.5
5	1.0	25	49.5	150.7	101.2	47	0.15	5	102.0	354.0	252.0
6	0.2	5	38.6	107.0	68.4	47	0.5	25	101.1	353.0	251.9
6	0.3	5	37.5	107.3	69.8						

TABLE V

Determination of Total Cholesterol in Serum by Windaus, Bloor, and New Methods

Serum No.	Windaus method	Bloor method		New method	
		Amount	Deviation from Windaus method	Amount	Deviation from Windaus method
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*per cent*
1	146.0	177	+21.2	138.1	−5.4
2	293.0	326	+11.3	286.4	−2.3
3	242.0	296	+22.3	238.2	−1.6
4	248.5	285	+14.7	243.3	−2.1
5	166.8	178	+6.7	162.7	−2.5
6	181.6			175.9	−3.1
7	192.2			193.2	+0.5
8	206.9			204.1	−1.4
9	267.8			272.6	+1.8

It must be remembered that digitonin precipitates practically all natural sterols, not all of which give color reactions. Colorimetric methods, therefore, yield reliable values only if cholesterol is the sole sterol present. Thus the gravimetric procedure of Windaus will give higher results than our method if saturated sterols such as dihydrocholesterol are present in the blood in relatively large amounts, as has been found in one case (4).

SUMMARY

An accurate, rapid, and relatively simple procedure for the determination of both free and combined cholesterol in 0.2 cc. of serum or blood has been presented.

We are indebted to Miss Klara Haager of Freiburg, Germany, and Dr. Mary E. Turner and Mr. V. A. Stoyanoff of this laboratory for much valuable assistance in this investigation.

BIBLIOGRAPHY

1. Schoenheimer, R., and Dam, H., *Z. physiol. Chem.*, **215**, 59 (1933).
2. Yoshimatsu, S., *Tohoku J. Exp. Med.*, 17, 147 (1931).
3. Obermer, E., and Milton, R., *Biochem. J.*, **27**, 345 (1933).
4. Schoenheimer, R., *Z. klin. Med.*, **123**, 749 (1933).

THE SYNTHESIS OF THEOPHYLLINE-5-METHYL-RHAMNOFURANOSIDE

By P. A. LEVENE and IRVING E. MUSKAT*

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York*)

(Received for publication, July 12, 1934)

The present communication is one of a series dealing with the synthesis of the naturally occurring nucleotides. For such synthesis it is required to develop methods first, for the preparation of the nucleosides and second, for phosphorylation of the latter in positions (3) or (5) of the ribose chain. Methods for phosphorylation in position (5) of the ribose residue have already been reported from this laboratory.[1,2] Nucleosides derived from pyranoses have been described by several workers but scarcely any attempts have been made to prepare furanosidic nucleosides. Such compounds are of special interest since all the naturally occurring nucleosides so far examined are of this type.

In the present investigation a rhamnofuranose derivative was chosen for condensation with theophylline, the reason being that 5-methyl-triacetyl-rhamnofuranose (VI) is readily converted into a crystalline 1-bromo-5-methyl-2,3-diacetyl-rhamnofuranose (VII). The theophylline condensation product (IX) was obtained in amorphous form but had the correct composition and did not reduce boiling Fehling's solution prior to acid hydrolysis.

The starting material for the preparation of 5-methyl-triacetyl-rhamnofuranose (VI) was the monoacetone rhamnose (I) first prepared by Fischer[3] and shown by Freudenberg[4] to have the furanose structure. The substance prepared by us had all the properties described by Freudenberg; thus the monomethyl monoacetone methylrhamnofuranoside (III) could be converted into a monomethyl rhamnose (IV) which, on oxidation, yielded methoxy lactic acid.

---

* National Research Fellow in Chemistry.

[1] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

[2] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 113 (1934).

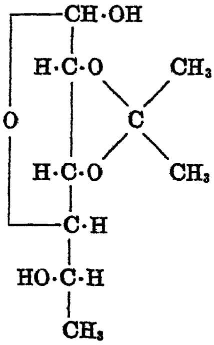
[3] Fischer, E., *Ber. chem. Ges.*, **28**, 1145 (1895).

[4] Freudenberg, K., *Ber. chem. Ges.*, **59**, 836 (1926).

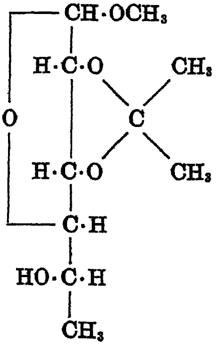
A monotosyl derivative (V) of monoacetone methylrhamnofuranoside (II) was prepared and this was shown not to react with sodium iodide under the conditions which convert primary alcoholic groups into iodides.

Of practical importance for future synthetic work on nucleotides was the possibility of the formation of orthoacetates of furanoses. Evidence was furnished that 5-methyl-rhamnofuranose (IV) forms a 1,2-methylorthoacetyl-3-acetyl-5-methyl-rhamnofuranose (VIII) although the substance has not yet been isolated in pure form.

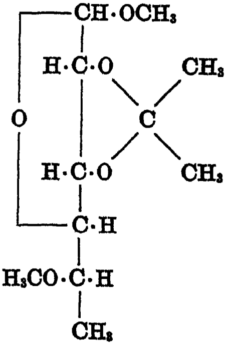
Incidentally, occasion was taken to compare the advantages of the methylation of the monoacetone methylrhamnofuranose by the method of Muskat[5] with those of the older methods of methylation, the result being favorable.



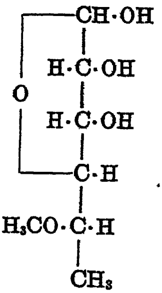
I



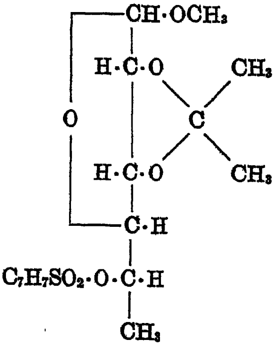
II



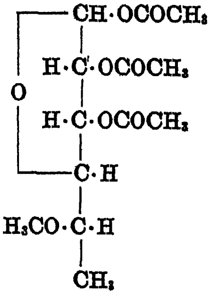
III



IV



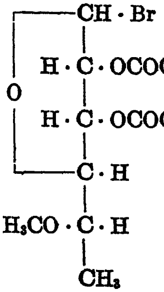
V



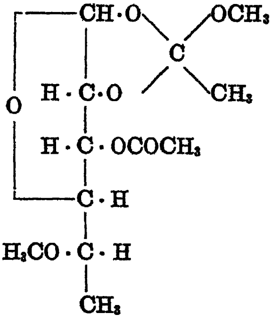
VI

---

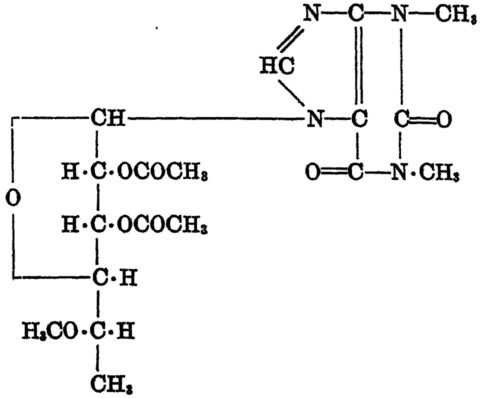
[5] Muskat, I. E., *J. Am. Chem. Soc.*, **56**, 693 (1934).



VII



VIII



IX

EXPERIMENTAL

Preparation of Monoacetone Rhamnofuranose (*I*)—Monoacetone rhamnofuranose was first prepared by Fischer[3] and its structure determined by Freudenberg.[4] Freudenberg showed that it existed in two forms: (1) m.p. 87–89°, $[\alpha]_D = 13° \rightarrow 17.8°$ (in water); (2) m.p. 79–80°, $[\alpha]_D = 10.9° \rightarrow 17.8°$ (in water). For this investigation the monoacetone rhamnofuranose was prepared as follows: 10 gm. of anhydrous rhamnose were suspended in 200 cc. of acetone (free of methyl alcohol) containing 0.2 per cent of sulfuric acid. Anhydrous copper sulfate (20 gm.) was added and the mixture was shaken at 37° for 20 hours. The mixture was filtered and the filtrate rendered neutral by shaking with calcium oxide. The calcium sulfate and excess calcium oxide were removed by means of filtration and washed with small quantities of acetone. The filtrate was evaporated under diminished pressure and the residual syrup was distilled. The entire product distilled without decomposition at about 115° under 0.2 mm. pressure. The product crystallized; m.p. 90°. The pure crystals did not reduce Fehling's solution.

Its specific rotation was

$$[\alpha]_D^{25} = \frac{+0.13° \times 100}{2 \times 1.015} = +6.4° \text{ (in water)}$$

$$[\alpha]_D^{25} = \frac{+0.36° \times 100}{2 \times 1.015} = +17.7° \text{ (in water with addition of ammonia)}$$

The substance had the following composition.

5.110 mg. substance: 9.935 mg. CO_2 and 3.610 mg. H_2O
$C_9H_{16}O_5$. Calculated. C 52.90, H 7.8
204.1 Found. " 53.01, " 7.9

Preparation of Monomethyl Monoacetone Methylrhamnofuranoside (III)—Freudenberg[4] first methylated monoacetone rhamnofuranose by the Purdie method. He obtained a product which distilled at 65–67° under 0.5 to 1 mm. pressure, $[\alpha]_D = -32.5°$ (in water).

This preparation was repeated, using 20.4 gm. of monoacetone rhamnofuranose, 100 gm. of methyl iodide, and 120 gm. of silver oxide. The methylation was twice repeated with half the above quantities of methylating agents and the product was isolated as a colorless, mobile syrup which distilled at 65° under 1 mm. pressure. Yield, 16.5 gm. (71 per cent).

Its specific rotation was

$$[\alpha]_D^{30} = \frac{-3.98° \times 100}{2 \times 3.704} = -53.7° \text{ (in methyl alcohol)}$$

The substance had the following composition.

5.179 mg. substance: 10.716 mg. CO_2 and 3.845 mg. H_2O
5.655 " " : 11.260 " AgI
$C_{11}H_{20}O_5$. Calculated. C 56.86, H 8.7, OCH_3 26.72
232.2 Found. " 56.42, " 8.3, " 26.28

Preparation of Monoacetone Methylrhamnofuranoside (II)—Monoacetone methylrhamnofuranoside was prepared by the method described above for monoacetone rhamnofuranose, except that 5 per cent of absolute methyl alcohol was added to the acetone. The entire product distilled without decomposition at 100–105° under 0.7 mm. pressure. Two fractions were collected. $n_D^{26} = 1.4487$ (first fraction), $n_D^{26} = 1.4520$ (second fraction).

The specific rotation of the first fraction was

$$[\alpha]_D^{26} = \frac{-3.09^\circ \times 100}{2 \times 2.315} = -66.7^\circ \text{ (in methyl alcohol)}$$

$$[\alpha]_D^{26} = \frac{-1.86^\circ \times 100}{2 \times 1.980} = -46.9^\circ \text{ (in water)}$$

The specific rotation of the second fraction was

$$[\alpha]_D^{26} = \frac{-1.85^\circ \times 100}{2 \times 1.766} = -52.4^\circ \text{ (in methyl alcohol)}$$

$$[\alpha]_D^{26} = \frac{-1.60^\circ \times 100}{2 \times 2.230} = -35.8^\circ \text{ (in water)}$$

Both fractions had the same composition and consisted of mixtures of α and β forms. The following analysis was obtained from the first fraction.

5.760 mg. substance: 11.650 mg. CO_2 and 4.300 mg. H_2O
3.070 " " : 3.319 " AgI
$C_{10}H_{18}O_5$. Calculated. C 55.02, H 8.3, OCH_3 14.22
218.1 Found. " 55.15, " 8.3, " 14.09

Methylation of Monoacetone Methylrhamnofuranoside—49 gm. of monoacetone methylrhamnofuranoside were methylated according to the method of Muskat.[5]

The potassium salt of monoacetone methylrhamnofuranoside is a white, crystalline substance. It is quite soluble in liquid ammonia and in anhydrous ether.

The salt reacts very violently with methyl iodide. It is therefore advisable to use ether as a diluent, and to add the methyl iodide (slightly in excess of the theoretical quantity) slowly and with constant shaking. The entire reaction was carried out at the temperature of an ice-salt bath. The reaction mixture was shaken for 10 minutes after all of the methyl iodide had been added. In order to insure that the reaction was complete and that all the potassium salt had reacted with the methyl iodide, a sample of the product was removed and dissolved in water. The aqueous solution was neutral, showing that the reaction had reached completion.

The ether was removed by evaporation and the reaction product was extracted several times with chloroform. The ether and chloroform extracts were combined, filtered, and the solvents removed by vaporization. The residual oil distilled completely at 65° under 0.1 mm. pressure. Yield, 48 gm. (92.3 per cent). $n_D^{26} = 1.4360$.

Its specific rotation was

$$[\alpha]_D^{26} = \frac{-4.94° \times 100}{2 \times 4.532} = -54.5° \text{ (in methyl alcohol)}$$

The substance had the same composition as, and is identical with, the monomethyl monoacetone methylrhamnofuranoside prepared by the methylation of monoacetone rhamnofuranose by the Purdie method described above.

Preparation of p-Toluenesulfonyl Monoacetone Methylrhamnofuranoside (*V*)—*p*-Toluenesulfonyl chloride (1.6 gm.) was added to a solution of acetone methylrhamnofuranoside (1 gm.) in dry pyridine (1.5 cc.). The mixture was allowed to stand overnight at room temperature and then dissolved in chloroform with the addition of water. The aqueous portion was extracted twice with small quantities of chloroform. The combined chloroform extracts were then washed successively with dilute hydrochloric acid, dilute sodium hydroxide, and water. The chloroform solution was now dried over anhydrous sodium sulfate, filtered, and the solvent removed by vaporization under diminished pressure.

The product was a pale yellow syrup which crystallized from methyl alcohol in white needles, m.p. 80°. The compound was very soluble in the usual organic solvents with the exception of ligroin.

Its specific rotation was

$$[\alpha]_D^{24} = \frac{-0.40° \times 100}{1 \times 3.270} = -12.2° \text{ (in } CH_3OH\text{)}$$

The substance had the following composition.

5.101 mg. substance: 10.200 mg. CO_2 and 2.755 mg. H_2O
10.050 " " : 6.505 " $BaSO_4$
$C_{17}H_{24}O_7S$. Calculated. C 54.80, H 6.5, S 8.60
372.26 Found. " 54.52, " 6.1, " 8.95

2 gm. of the *p*-toluenesulfonyl monoacetone methylrhamnofuranoside were heated in a sealed tube for 2 hours with 2 gm. of sodium iodide and 10 cc. of acetone at 100°. The solution was then evaporated to dryness under diminished pressure, and the dry residue extracted repeatedly with hot chloroform. The chloroform extracts were washed with dilute sodium thiosulfate solution and then with water. The chloroform extract was dried and the solvent removed by vaporization.

The resulting oil (2 gm.) was crystallized from methyl alcohol and melted at 80°. A mixture with the original *p*-toluenesulfonyl monoacetone methylrhamnofuranoside also melted at 80°. A qualitative test for halogen was negative. This is in accord with previous observations that a *p*-toluenesulfonyl group attached to a secondary alcoholic group cannot be replaced under these conditions.

Preparation of Monomethyl Rhamnofuranose (*IV*)—The monomethyl monoacetone methylrhamnofuranoside (14 gm.) was hydrolyzed with 1.5 per cent sulfuric acid (300 cc.) for 90 minutes at the boiling point (according to the method of Freudenberg[4]). All the monomethyl monoacetone methylrhamnofuranoside had dissolved within that time. The solution was rendered neutral with barium carbonate, filtered, and the water removed under diminished pressure, at a temperature not exceeding 40°. The residual syrup was dissolved in acetone and filtered from barium salts. The acetone was removed by vaporization under diminished pressure and a syrup similar to that described by Freudenberg was obtained.

A sample of the syrupy monomethyl rhamnofuranose was oxidized with silver oxide according to the method of Freudenberg.[4] The silver salt of methoxylactic acid was isolated exactly as described by him.

Preparation of Triacetyl-Monomethyl-Rhamnofuranose (*VI*)—10 gm. of monomethyl rhamnofuranose were dissolved in a solution of 20 cc. of acetic anhydride in 25 cc. of pyridine. The solution was allowed to stand at 0° overnight and then poured onto ice with shaking.

The ice-cold aqueous solution was extracted several times with small quantities of chloroform and the combined chloroform extracts were washed with dilute sulfuric acid, sodium hydroxide, and finally with water. The chloroform solution was then dried over anhydrous sodium sulfate and the solvent removed under diminished pressure.

The residual syrup distilled almost completely at 128-131° under 2 mm. pressure. On standing, it crystallized in part. The crystals were separated and recrystallized from methyl alcohol. They melted at 114°.

The specific rotation of the solid was

$$[\alpha]_D^{25} = \frac{-0.45^\circ \times 100}{2 \times 0.340} = -66.2^\circ \text{ (in methyl alcohol)}$$

The specific rotation of the liquid was

$$[\alpha]_D^{25} = \frac{-0.58^\circ \times 100}{1 \times 2.06} = -28.1^\circ \text{ (in methyl alcohol)}$$

The crystalline form must have the α structure since in the *l* series the difference between the rotatory values of $\alpha - \beta$ should be negative.[6] Both the solid and liquid forms had the same composition. The following analysis was obtained for the crystalline product.

4.585 mg. substance: 8.555 mg. CO_2 and 2.570 mg. H_2O
7.695 " " : 6.065 " AgI
110.8 " " required 11.05 cc. 0.1 N NaOH
$C_{13}H_{20}O_8$. Calculated. C 51.26, H 6.6, OCH_3 10.20, $COCH_3$ 42.43
304.2 Found. " 51.06, " 6.3, " 10.40, " 42.80

A portion of the triacetyl-methyl-rhamnofuranose was deacetylated with barium methylate according to the method of Levene and Tipson.[7] An effort was then made to crystallize the 5-methyl rhamnose, but without success.

Preparation of Bromo-Diacetyl-Monomethyl-Rhamnofuranose (VII)—10 gm. of triacetyl-monomethyl-rhamnofuranose were mixed with 50 cc. of glacial acetic acid containing 40 per cent of dry hydrogen bromide.[8] The resulting solution was allowed to stand at room temperature for 60 minutes, after which the hydrogen bromide gas was removed under diminished pressure at room temperature. The solution was then diluted with 100 cc. of toluene and evaporated to a thick syrup under diminished pressure at 35°. Two further portions of 50 cc. of toluene were run in and evaporated off. This syrup was now dissolved in 50 cc. of benzene and the solution was evaporated to a thick syrup. Traces of solvent were removed under a high vacuum at 35°.

---

[6] Hudson, C. S., *J. Am. Chem. Soc.*, 31, 66 (1909).

[7] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, 93, 631 (1931).

The resulting thick, pale yellow syrup was dissolved in dry ether, a little charcoal was added, the mixture shaken and filtered through a fluted filter, the filtrate obtained being faintly yellow. The filtrate was then concentrated by evaporating the ether under suction. The rapid evaporation of the ether cooled the solution and crystallization set in. The crystals were collected on a filter and washed with a mixture of ether and pentane (1:1). The colorless crystals melted at 100.5°.

The substance had the following composition.

8.570 mg. substance: 4.690 mg. AgBr (direct precipitation)
$C_{11}H_{17}O_6Br$. Calculated. Br 24.6
325.05 Found. " 23.3

Its specific rotation was

$$[\alpha]_D^{24} = \frac{-2.15^\circ \times 100}{2 \times 0.610} = -176.2^\circ \text{ (in dry chloroform)}$$

Preparation of Monomethyl-Diacetyl-Theophylline-Rhamnofuranoside (IX)—2 gm. of bromo-diacetyl-monomethyl-rhamnofuranose were dissolved in 100 cc. of dry xylene. 2 gm. of the silver salt of theophylline, dried to constant weight at 140° under reduced pressure, were added and the mixture was kept at about 90° overnight, after which no bromine was found in a small filtered test portion.

The mixture was filtered, and the almost colorless filtrate was shaken with charcoal and filtered again. On standing overnight at room temperature a small amount of theophylline was deposited.

---

[8] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **92**, 109 (1931).

The filtrate from this was poured into 2 liters of ligroin and the precipitate collected and dried. It has no definite melting point. It shrinks at about 62° and melted at about 83°. This is charteristic of similar theophylline derivatives.

Its specific rotation is

$$[\alpha]_D^{24} = \frac{-0.55° \times 100}{2 \times 1.184} = -23.2° \text{ (in methyl alcohol)}$$

The substance had the following composition.

5.190 mg. substance: 9.625 mg. CO_2 and 2.730 mg. H_2O
1.940 " " : 0.215 cc. N (757 mm., 29°)
$C_{18}H_{24}O_8N_4$. Calculated. C 50.94, H 5.66, N 13.2
424.1 Found. " 50.60, " 5.88, " 12.5

Preparation of 1,2-Methylorthoacetyl-3-Acetyl-5-Methyl-Rhamnofuranose (VIII)—The orthoacetate was prepared according to the method of Haworth.[9] 1 gm. of the bromo-diacetyl-monomethylrhamnofuranose was dissolved in dry methyl alcohol containing 2 cc. of quinoline and the solution allowed to stand in the refrigerator during 3 hours. 50 cc. of chloroform were then added and the quinoline was removed by shaking with dilute sulfuric acid. The chloroform extract was now washed with sodium bicarbonate and finally with water and dried over anhydrous sodium sulfate. The chloroform was removed by evaporation and the residual colorless oil was kept under a high vacuum overnight. $n_D^{24} = 1.4490$.

The specific rotation was

$$[\alpha]_D^{24} = \frac{+0.75° \times 100}{2 \times 1.188} = +31.5° \text{ (in methyl alcohol)}$$

The substance had the following composition.

4.600 mg. substance: 8.800 mg. CO_2 and 2.845 mg. H_2O
5.495 " " : 9.000 " AgI
124.6 " " required 6.75 cc. 0.1 N NaOH
$C_{12}H_{20}O_7$. Calculated. C 52.1, H 7.3, OCH_3 22.4
276.1 Found. " 52.16, " 6.9, " 21.6
Calculated. $COCH_3$ 15.6 for one acetyl group
" 31.2 " two " groups
Found. " 23.2

---

[9] Haworth, W. N., *J. Chem. Soc.*, 2862 (1931).

The substance was therefore a mixture of the glycoside and the orthoacetate and no effort was made to separate them.

We wish to thank Dr. R. S. Tipson for his kind assistance in the preparation of the manuscript.

SYNTHESIS OF THE HEXURONIC ACIDS

VII. THE SYNTHESIS OF *l*-GLUCURONIC ACID AND THE RESOLUTION OF *dl*-GALACTURONIC ACID*

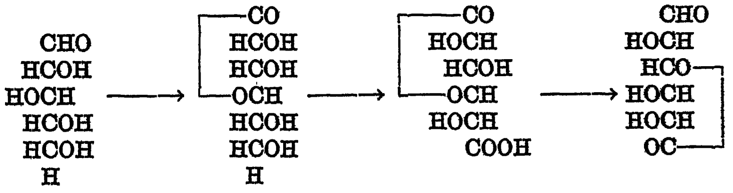
BY CARL NIEMANN AND KARL PAUL LINK

(*From the Biochemistry Research Laboratory, Department of Agricultural Chemistry, University of Wisconsin, Madison*)

(Received for publication, May 17, 1934)

INTRODUCTION

In 1891 Fischer and Piloty reported the synthesis of *d*-glucuronic acid (1). These investigators reduced a solution of *d*-saccharic acid lactone with sodium amalgam and isolated crystalline *d*-glucurone in about a 1 per cent yield. The general method of partial reduction of a dicarboxylic acid lactone to the corresponding uronic acid has been applied by us recently to the synthesis of *d*- and *l*-mannuronic acids, *dl*-galacturonic acid, and *dl*-alluronic acid (2–5). In this communication we report the synthesis of *l*-glucuronic acid by the same general procedure. The reactions involved in the synthesis are illustrated by the following structural formulæ.



d-Xylose γ-*d*-Gulonolactone γ-*l*-Saccharolactone (not isolated) *l*-Glucurone

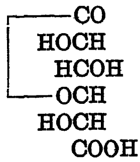
The above formulæ suggest several points that deserve discussion. It has been pointed out by Tollens (6) that *d*-saccharic

---

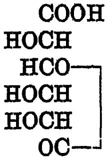
* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by grants from the University Research Fund.

acid monolactone may exist in two isomeric forms, which are designated simply as α and β. Similarly for *l*-saccharic acid we may expect the same number of isomeric monolactones; *e.g.*,



and



α-γ-*l*-Saccharolactone β-γ-*l*-Saccharolactone

It is evident that, upon partial reduction, the α-lactone will yield *l*-glucuronic acid, and the β-lactone *d*-guluronic acid. This raises the question as to whether or not an aqueous solution of saccharic acid will contain both isomeric monolactones. The isolation of *d*-glucurone (1) is given as evidence for the existence of the α-monolactone in the case of *d*-saccharic acid (6). Similarly in this research we can consider the isolation of *l*-glucurone to be indicative of the existence of the α-monolactone of *l*-saccharic acid.

Direct evidence for the presence of the β-monolactone in a solution of either *d*- or *l*-saccharic acid is lacking, but there remains the possibility that it may exist and that *l*- or *d*-guluronic acid is formed in conjunction with *d*- or *l*-glucuronic acid when an aqueous solution of *d*- or *l*-saccharic acid is subjected to reduction with sodium amalgam. At present it is impossible to decide this question because of the lack of conclusive experimental observations.

The observed formation of γ-*d*-gulonolactone during the synthesis of *l*-glucurone is obviously the result of an overreduction of the uronic acid. The presence of the aldonolactone contributes to the difficulty encountered in obtaining crystalline *l*-glucurone but it may not be the only factor responsible for the low yields obtained in this synthesis. Fischer and Piloty (1) state that the synthesis of *d*-glucurone from *d*-saccharic acid cannot be considered as a practical method of preparation. Our experience with the *l* compound is in complete accord with this view.

The values reported for the melting point of *d*-glucurone vary from 167–180°. Due to the fact that the compound decomposes slowly before the melting point is reached, the rate of heating is a very important factor. The value reported herewith for the melting point of *l*-glucurone was obtained when the temperature of the bath was raised at the rate of 5–6° per minute. More rapid heating produced higher melting points.

The synthesis of *dl*-galacturonic acid from mucic acid was reported by us recently (4). At that time an effort was made to resolve the *dl* acid through the fractional crystallization of brucine *dl*-galacturonate. This and later attempts, with the same technique, met with only partial success. Whereas, it was possible, through the fractionation of the brucine salts, to obtain the *d* acid in a state of optical purity, it was impossible to prepare the *l* acid in an uncontaminated condition.[1]

It is generally recognized that a racemate possesses physical properties that are different from those exhibited by the active forms (7). Thus under conditions favorable to racemate formation it should be possible to separate a partially resolved acid into a racemate and an active form. This situation has been realized and we have been able to obtain optically pure *l*-galacturonic acid from a mixture containing 35 per cent of the *d* acid and 65 per cent of the *l* acid by fractional crystallization from 75 per cent ethyl alcohol.

l-Galacturonic acid, its oximehydroxylamine salt, and its diacetone compound have been described previously (8). As an additional representative derivative of *l*-galacturonic acid we now report the preparation and properties of α-methyl-*l*-galacturonide methyl ester.[2] In addition we have prepared a methylglycoside methyl ester of *dl*-galacturonic acid and have determined its properties.

EXPERIMENTAL

The methods used for the determination of the melting points and rotations have been described in this *Journal* (10). The exact experimental details in the preparative work are given only where the general technique has not been previously reported in papers published either by ourselves or others. All analyses were made with the Pregl micromethods.

---

[1] Repeated recrystallization from five solvents were unsuccessful. Mixtures containing from 30 to 40 per cent of the *d* acid were invariably obtained.

[2] The system of nomenclature used in this case is that of Hudson (9), wherein the isomer possessing the highest rotation, either dextro or levo, is designated as the α form. This arbitrary designation has no structural implications.

Preparation of Calcium l-Saccharate—Crystalline *d*-xylose was converted into crystalline γ-*d*-gulonolactone by the method of Fischer and Stahel (11).[3] Following the directions described by the same authors the aldonolactone was oxidized with nitric acid and the dicarboxylic acid isolated as the calcium salt.

Analysis—$C_6H_8O_8Ca \cdot 4H_2O$.	Calculated.	Ca 12.50
	Found.	" 12.45

Preparation of Crude Barium l-Glucuronate—35 gm. of the above calcium salt were dissolved in sufficient hot water and the calcium removed by the addition of the requisite quantity of oxalic acid. The aqueous solution of *l*-saccharic acid was then concentrated under reduced pressure to a thick syrup which was heated on a boiling water bath for 4 to 5 hours. The colored syrup was diluted to 600 cc. and reduced with 2.5 per cent sodium amalgam in the usual manner (1–3, 13). The hexuronic acid present in the reaction mixture was then converted into the barium salt (2, 3) and isolated in this state. The yield was 9.0 gm.

Rotation—$[\alpha]_D^{25} = -8.5° \pm 1.0°$ (in water, $c = 0.73$ per cent).

Analysis—$(C_6H_9O_7)_2Ba$.	Calculated.	Ba 26.30, CHO 11.10
	Found.	" 25.60, " 9.00

Preparation of l-Glucurone—8.0 gm. of the crude barium salt were converted into the lactone in the same manner as described for the preparation of *d*- and *l*-mannuronolactone (2, 3) with the exception that the barium was removed as the oxalate instead of the sulfate. The first crystalline substance obtained (0.30 gm.) did not have reducing properties when purified, and possessed a melting point of about 183°. Upon the removal of this material and after standing for a period of several weeks in the presence of acetone a small quantity (0.20 gm.) of *l*-glucurone crystallized from the residual syrup. This crude product was contaminated with a small amount of the previously isolated non-reducing substance. The compound was purified by two recrystallizations from hot water and ethyl alcohol and dried over phosphorus pentoxide for 1 week prior to the determination of its characteristic properties and analytical constants.[4]

---

[3] Later, while engaged in obtaining a further quantity of *l*-glucurone, the method of La Forge (12) was used for the preparation of this intermediate.

Melting Point—The lactone sintered and darkened at about 165° and melted with decomposition between 169–172°.

Rotation—$[\alpha]_D^{25} = -18.5° \pm 1.0°$ (in water, $c = 0.6$ per cent).

Analysis—$C_6H_8O_6$. Calculated. C 40.92, H 4.55
Found. " 40.99, " 4.90

Characterization of Non-Reducing Substance—The crystalline non-reducing substance obtained during the isolation of *l*-glucurone was recrystallized from 95 per cent ethyl alcohol and dried over phosphorus pentoxide at room temperature for several days. The compound so prepared had a melting point of 183–184° and possessed an $[\alpha]_D^{25}$ of $-54.0°$ (in water), indicating that the compound in question was γ-*d*-gulonolactone. Additional evidence was found to verify this contention. A mixed melting point with an authentic specimen of the aldonolactone showed no depression.

Preparation of dl-Galacturonic Acid—*dl*-Galacturonic acid was prepared by the method of Niemann and Link (4) with the following important modifications.[5] After the reduction of the mucic acid lactone with sodium amalgam the solution was adjusted to neutrality with sodium hydroxide and concentrated to a small volume. The organic acids were liberated by the addition of the requisite quantity of sulfuric acid (5 N) and the sodium sulfate removed from the concentrate by the addition of 10 to 12 volumes of 95 per cent ethyl alcohol. From this stage the barium salt was isolated as previously described in other communications from this laboratory (2, 3, 5). The barium *dl*-galacturonate was converted into the free acid by the usual method (4, 14) and was obtained as the monohydrate.

Melting Point—The acid sintered at 110° and melted with decomposition at 156–158°.

---

[4] The aldehydic nature of the compound was demonstrated by the reduction of Fehling's solution when heated with that reagent and by its ability to reduce the alkaline hypoiodite reagent.

[5] As previously cited (5) we have abandoned the use of basic lead acetate for purposes of intermediate isolation and have resorted to the technique described by Fischer and Piloty (1).

Analysis

$C_6H_{10}O_7 \cdot H_2O$.	Calculated.	Neutralization equivalent	47.20 cc.	0.1 N alkali
	Found.	" "	48.00 "	0.1 " "

Isolation of d-Galacturonic Acid—10.0 gm. of *dl*-galacturonic acid monohydrate were dissolved in 50 cc. of 90 per cent ethyl alcohol and added to a solution of 20.0 gm. of brucine dihydrate in 50 cc. of the same solvent. After standing overnight in the ice chest the crystalline precipitate was collected (about 28 gm.) and recrystallized from 150 cc. of a 70 per cent acetone water mixture. The product was again recrystallized from 200 cc. of 75 per cent acetone and after washing freely with methyl alcohol and chloroform was dried at room temperature over phosphorus pentoxide. The preparation (10 gm.) melted at 180–181° with decomposition.

12.0 gm. of the brucine salt were dissolved in 100 cc. of water and 100 cc. of saturated barium hydroxide added to the cold solution. The brucine was removed by filtration and extraction with chloroform. The excess alkali was then neutralized with carbon dioxide, the solution heated at 60° for 30 minutes, the barium carbonate removed, and the barium salt of the uronic acid isolated from the filtrate in the usual manner (14). The yield was about 5 gm. and the salt when thoroughly dried at 78° under 1 mm. pressure had an $[\alpha]_D^{25}$ of $+25.8°$.

4.0 gm. of the above barium salt were converted into the free acid (4, 14) and isolated as the monohydrate (1.9 gm.).

Melting Point—The acid sintered at 110° and melted with decomposition at 157–159°.

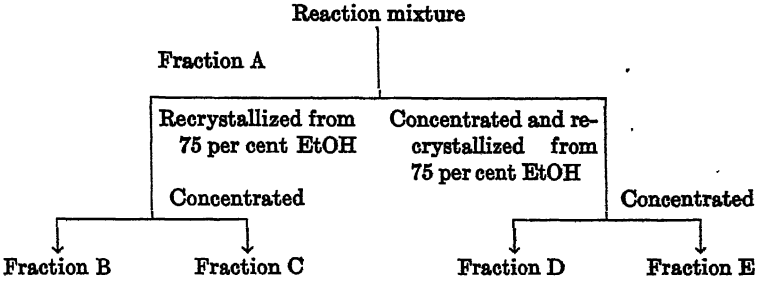
Rotation—$[\alpha]_D^{25} = +50.0° \pm 2.0°$ (in water, $c = 1.36$ per cent).

Analysis

$C_6H_{10}O_7 \cdot H_2O$.	Calculated.	Neutralization equivalent	47.20 cc.	0.1 N alkali
	Found.	" "	48.50 "	0.1 " "

Isolation of l-Galacturonic Acid—The mother liquors resulting from the isolation and recrystallization of brucine *d*-galacturonate (above) were combined and concentrated, whereupon a brucine salt (7.0 gm.) of melting point 162–163° was isolated. 12.0 gm. of this salt upon treatment with barium hydroxide yielded 5.0 gm. of a barium salt which had an $[\alpha]_D^{25}$ of $-7.0°$. 4.26 gm. of this barium salt (35 per cent *d*- and 65 per cent *l*-) were treated with

14.20 cc. of N sulfuric acid. The uronic acid was isolated from the reaction mixture in the usual way (14) and fractionated according to the following scheme. All crystallizations were carried out at 25°.



The yields and rotations of the various fractions are given in the accompanying tabulation.

Fraction	Yield	Rotation $[\alpha]_D^{23}$ in water
	mg.	*degrees*
B	1400	−5.0 ± 1.0
C	70	−40.0 ± 2.0
D	30	−52.0 ± 2.0
E	15	−43.0 ± 2.0

From the tabulation it is evident that Fraction D is an optically pure specimen of *l*-galacturonic acid. Fractions C and E can be combined and recrystallized to yield a further quantity of the *l* acid. A theoretical yield requires the isolation of 2.1 gm. of the racemic acid and 0.9 gm. of the levo acid. The following are constants exhibited by Fraction D.

Melting Point—The acid melts with decomposition between 156–158°.

Rotation—$[\alpha]_D^{23} = -52.0° \pm 2.0°$ (in water, $c = 0.18$ per cent).

Analysis

$C_6H_{10}O_7$.	Calculated.	Neutralization equivalent	51.52 cc. 0.1 N alkali
	Found.	" "	51.30 " 0.1 " "

Preparation of α-Methyl-l-Galacturonide Methyl Ester—0.13 gm. of dry *l*-galacturonic acid was treated with 50 cc. of 0.5 per cent methyl alcoholic hydrogen chloride and converted into the methyl ester methylglycoside in the usual manner (15–17). The crude product was purified by recrystallization from 95 per cent ethyl alcohol and dried for 1 week at room temperature over phosphorus pentoxide prior to analysis. The yield was 0.01 gm.

Melting Point—The compound melted gradually between 130–135°.

Rotation—$[\alpha]_D^{25}$ = about $-120°$[6] (in water, c = 0.3 per cent).

Preparation of Methyl Ester Methylglycoside of dl-Galacturonic Acid—0.92 gm. of dry *dl*-galacturonic acid was transformed into the ester glycoside by the general procedure described above. The product obtained from the reaction mixture was recrystallized from 95 per cent ethyl alcohol and dried for 24 hours at 78° under 1 mm. pressure over phosphorus pentoxide prior to analysis. The yield was 0.2 gm.

Melting Point—The compound melted between 126–128°.

Analysis—$C_8H_{14}O_7$.	Calculated.	OCH_3	27.93
	Found.	"	27.70

SUMMARY

1. Crystalline *l*-glucurone has been synthesized by the partial reduction of *l*-saccharic acid lactone and its properties are in accord with those of its previously known enantiomorph.
2. *dl*-Galacturonic acid has been resolved into its optically active components.
3. The preparation of α-methyl-*l*-galacturonide methyl ester and a methyl ester methylglycoside of *dl*-galacturonic acid has been described.

We wish to thank Dr. Eugene Schoeffel for conducting the microanalyses and Dr. Sam Morell for raising the question as to the existence of the isomeric monolactone of saccharic acid.

BIBLIOGRAPHY

1. Fischer, E., and Piloty, O., *Ber. chem. Ges.*, 24, 521 (1891).
2. Niemann, C., and Link, K. P., *J. Biol. Chem.*, 100, 407 (1933).

---

[6] This value must be considered as only approximate, because of the extremely small sample (3 mg.) used for the determination.

3. Niemann, C., McCubbin, R. J., and Link, K. P., *J. Biol. Chem.*, **104**, 737 (1934).
4. Niemann, C., and Link, K. P., *J. Biol. Chem.*, **95**, 203 (1932).
5. Niemann, C., Karjala, S., and Link, K. P., *J. Biol. Chem.*, **104**, 189 (1934).
6. Tollens, B., Kurzes Handbuch der Kohlenhydrate, Leipsic, 3rd edition (1914).
7. Werner, A., Lehrbuch der Stereochemie, Jena (1904).
8. Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 743 (1934).
9. Hudson, C. S., Rapports sur les hydrates de carbon, Dixieme Conference de l'Union Internationale de Chemie, Liége (1930).
10. Niemann, C., Schoeffel, E., and Link, K. P., *J. Biol. Chem.*, **101**, 337 (1933).
11. Fischer, E., and Stahel, R., *Ber. chem. Ges.*, **24**, 528 (1891).
12. La Forge, F. B., *J. Biol. Chem.*, **36**, 347 (1918).
13. Kiliani, H., *Ber. chem. Ges.*, **58**, 2344 (1925).
14. Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 195 (1934).
15. Morell, S., and Link, K. P., *J. Biol. Chem.*, **100**, 385 (1933).
16. Ehrlich, F., and Guttmann, R., *Ber. chem. Ges.*, **66**, 220 (1933).
17. Smolenski, K., and Cichocki, M., *Collect. Czechsl. Chem. Communicat.*, **4**, 500 (1932); *Chem. Abst.*, **27**, 1618 (1933).

AN ULTRAMICROMETHOD FOR THE DETERMINATION OF THE OXYGEN CONTENT OF BLOOD

By JOHN S. DONAL, Jr.

(*From the Laboratory of Pharmacology and the Johnson Foundation for Medical Physics, School of Medicine, University of Pennsylvania, Philadelphia*)

(Received for publication, July 10, 1934)

The desire to make accurate estimations of oxygen in very small amounts of blood has led to the development of numerous methods to accomplish this end. For example, the oxygen in 0.05 cc. of blood has been estimated by Krogh (1919), employing a modification of the spectroscopic method of Hartridge (1912). Recently Mook (1930, 1931), using a modification of the principles introduced by Van Slyke (1917) and by Van Slyke and Neill (1924), has determined the oxygen content of blood samples from 0.05 to 0.07 cc. in volume.

The observation that positive ions are formed at the surface of a heated tungsten filament when in an atmosphere containing traces of oxygen (Donal, 1930) suggested that a method might be devised for accurately estimating amounts of this gas in much smaller samples of blood, water, or any solution from which it could be quantitatively extracted. This principle being used, a method has been developed to determine the oxygen in quantities of the order of 0.001 cc. of blood. Compared with the results of determinations of large samples of the same blood by the method of Van Slyke and Neill (1924), the mean difference is −0.16 volume per cent and the average deviation of the results from this difference is 0.56 volume per cent.

Method

Measurement of the sample to be analyzed and of the reagent used to facilitate liberation of oxygen is accomplished by an adaptation of the capillary tube technique of Richards, Bordley, and Walker (1933). A fraction of the gases extracted from the sample of blood is admitted to an evacuated chamber containing a hot tungsten filament. The positive ions formed at the filament surface are attracted to a negatively charged collecting electrode. The resulting current is amplified and measured by means of a sensitive galvanometer. To transfer this reading into volumes of oxygen released by the blood it is only necessary to repeat the procedure with a sample of a gas of known oxygen content. Air is ideally suited to this purpose.

It has been demonstrated that the initial deflection of a galvanometer of the d'Arsonval type is directly proportional to the potential applied to the instrument, when this potential decreases exponentially with the time and the negative exponent is a constant (Laws, 1917). It can be shown mathematically that in such experiments as are described below the initial deflection of the galvanometer is directly proportional to the quantity of oxygen introduced into the chamber containing the hot tungsten filament. The results of the experiments confirm this.

Apparatus

The apparatus in which the gases are released and brought in contact with the tungsten filament, together with the arrangements for measuring the positive ions, is illustrated in Fig. 1. It consists of a series of glass chambers separable from one another by glass stop-cocks and connected at two points to the vacuum pumps by tubes equipped with stop-cocks. The pumps used were a Megavac (Central Scientific Company) and a single stage mercury vapor diffusion pump. Pressures as low as 10^{-5} mm. of mercury were readily obtainable. Measurements of pressure were made with the usual type of McLeod gage, the one employed being calibrated from 5×10^{-2} to 1×10^{-6} mm. of mercury.

Chamber *A* is the space in which the gas is initially released. As soon as this is effected, chamber *B* is connected with *A* and the gas distributes itself between the two. *B* is then disconnected from *A* and connected with the drying tube *C* and the chamber beyond it by rotating the plug through 180°. Then, on closing stop-cock *J* a portion of the gas is isolated in chamber *D*. From this it is admitted into the space containing the tungsten filament and the positive ions formed at the filament surface are collected by a negatively charged electrode. The resulting current, after amplification, deflects a sensitive galvanometer.

The small chamber D' permits the introduction into the filament

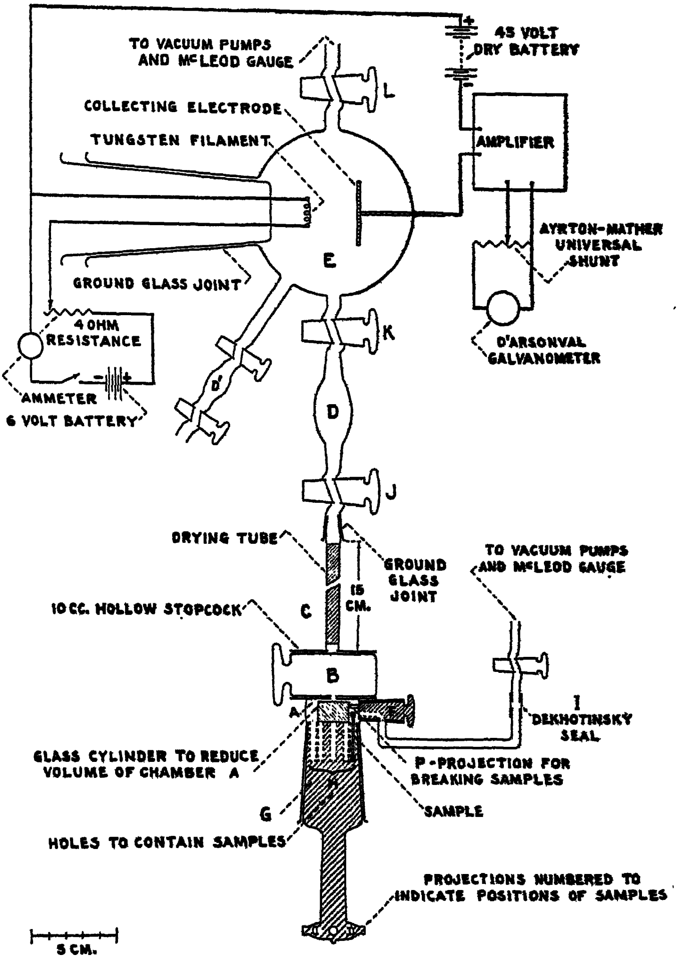


FIG. 1. Apparatus for the determination of oxygen in small samples of blood. The holes *H* to hold the samples are eight in number, on the circumference of a circle. The joint at the top of chamber *C* and the De Khotinsky seal indicated at *I* permit the removal of the lower portion of the apparatus for replacement of the reagents used for desiccation.

chamber of any gas mixture desired for experimental purposes. It is used only for control experiments.

Details—*A* is the space between the upper surface of the glass plug *G* and the sleeve of the hollow stop-cock *B*. When the stop-cock *F* is in the position illustrated in Fig. 1, *A* or both *A* and *B* can be exhausted separately from the rest of the apparatus through the passage in *F*. The sample of blood to be analyzed or of air for calibration is contained in a sealed capillary glass tube placed upright in one of the holes drilled into the upper surface of plug *G*. After the plug with its capillary tube or tubes has been tightly fitted into its sleeve, and after the chamber *A* has been completely exhausted, stop-cock *F* is turned, a projection on it breaks the capillary, and the contained gases are released.

The hollow stop-cock *B* serves to receive a known part of the extracted gases. When turned it transfers its contents into the chambers above and at the same time isolates the gases to be analyzed from contact with the remainder of the blood sample, the fluid part of which, evaporating into the vacuum, would otherwise soon saturate the desiccators. In order to increase the fraction of the extracted gases received by *B*, it was of advantage to make the volume of chamber *A* as small as possible. To this end a solid glass cylinder was cemented to the top surface of plug *G*, as indicated in Fig. 1.

The drying tube contains a column of anhydrous magnesium perchlorate (Willard and Smith, 1922) 5 cm. long and a column of phosphorus pentoxide 7 cm. long. If desired the stop-cock *J* permits any portion of the gas from *B* to be held in the drying tube, slowing the initial rush of gas from *B* into the evacuated chamber *D*.

Chamber *D* permits the isolation of a portion of the dried gases. The closure of stop-cock *J* before admission of the contents of *D* into the filament chamber prevents uncertainties arising from variations in the rate of flow of additional gas from *B* through the desiccator.

The tungsten filament used in chamber *E* has a surface area of approximately 60 sq. mm. The ions formed at its surface are attracted to a flat circular nickel electrode which is maintained at a potential of 45 volts below that of the filament by means of dry batteries. In order to facilitate replacement after extensive oxidation the tungsten filament is mounted on a removable vacuum-tight joint.

A vacuum tube current amplifier, of the type described by Razek and Mulder (1929), is connected as shown in the figure and used to magnify the current resulting from the collection of positive ions by a factor of from 2 to 20 times. A reflecting d'Arsonval galvanometer,[1] protected by an Ayrton-Mather universal shunt, is connected to the output terminals of the current amplifier. The current sensitivity of the galvanometer is approximately 1×10^{-9} amperes per mm. of deflection at 1 meter distance. The period (9.6 seconds) was found by experiment to be approximately 6 times the duration of the measurable ion collection for the largest samples used.

Procedure

Reagents—In preliminary experiments it was found that the extraction of oxygen from unlaked blood was incomplete. Therefore the blood was treated with saponin and potassium ferricyanide before exposure to the vacuum. Extraction was found to be complete after this treatment and the reagent now employed is that of Van Slyke and Neill (1924) with the exceptions that only half the prescribed amount of water is added and, as foaming is not a factor, the octyl alcohol is omitted. As it is impracticable to extract the oxygen dissolved in the reagent, it is saturated with air before being used. Water introduced into the apparatus with the air sample is similarly treated. Oxygen is assumed to have the same solubility in the reagent as in the distilled water, and the amount of oxygen dissolved in each is calculated from the Landolt-Börnstein solubility tables (1923). These quantities of oxygen are subtracted from the totals found to secure the oxygen actually present in the blood and air. The exact procedure will be illustrated in a sample calculation.

Measurement of Samples—The oxygen content of a blood sample is determined by comparing the galvanometer deflection yielded by the blood with the deflection obtained from a sample of air introduced under the same conditions. Since the oxygen content of the blood is proportional to the *ratio* of the galvanometer deflections per unit volume of blood and per unit volume of air, only the ratio of the sample volumes is essential.

---

[1] Manufactured by the Leeds and Northrup Company.

The samples of blood and of air are prepared in adjacent portions of the same piece of mechanically drawn glass tubing,[2] of approximately 0.6 mm. internal diameter. Pieces of this tubing 60 cm. long were broken into many short sections and the internal diameters of the sections measured with a dividing engine. In no case did the diameter of any one section of a 60 cm. piece vary more than 1 per cent from the average of all measurements on portions of that piece. This uniformity permits the calculation of the ratio of the volumes of the samples from measurements of their lengths alone.

The lengths, 2 to 4 mm., of the columns enclosed in the capillary tubes are measured by fastening each sample with wax to the same portion of a steel scale engraved in 0.25 mm. divisions, and reading the lengths with the aid of a microscope having a 10-fold magnification. Measurements of column lengths were made between planes tangent to the surfaces of all menisci. The lengths thus measured were corrected by a distance equal to one-third the height of each meniscus. Repeated measurements of the same column seldom differ by more than 1 per cent.

Preparation of Samples—The blood was delivered under oil, from the syringe in which it was collected, to a small beaker. A sample is taken into a capillary pipette and transferred to a mercury-filled capillary tube in such a way that it is protected from exposure to air by mercury at either end of the column. An approximately equal volume of the reagent is similarly introduced into the capillary, separated from the blood and from outside air by mercury. The ends of the tube are sealed with De Khotinsky cement and, after measurement of the lengths of the columns, the blood and reagent are mixed by centrifuging. The capillary tube containing air, and a small quantity of water to insure saturation of the air with water vapor, is prepared in a similar manner.

Details of Preparation of Samples—The syringe ("water manipulator") described by Richards, Bordley, and Walker (1933) was filled with mercury and attached by rubber tubing to a piece, about 30 cm. long, of the 0.6 mm. capillary tube. By turning the micrometer screw the piston of this syringe is advanced so that the rubber tubing and the capillary are filled with mercury. The mercury column is then withdrawn about 1 cm. from the tip of the capillary to make room for the introduction of the blood sample. A pipette with a tip of about 0.15 mm. outside diameter and several cm. long is fitted with a rubber tube for the application of pressure by mouth. Blood, preceded and followed by droplets of oil to exclude air, is then withdrawn into the pipette from a sample under oil and the tip of the pipette is introduced into the open end of the capillary and held close to the mercury meniscus. Enough blood is ejected to fill the open end of the capillary and to expel from the capillary the oil drop initially in the tip of the pipette. (If this droplet of oil is allowed to remain in the end of the capillary it is very difficult to prevent its inclusion in the final blood column, which results in an uncertainty in the measurement of the column length.) As this procedure exposes the blood in the open end of the capillary to air, this portion is discarded immediately by introducing a pipette filled with mercury and displacing all but about 3 mm. of the blood with mercury. The capillary now contains the blood sample sealed at each end with mercury.

---

[2] Manufactured by the International Resistance Company, Philadelphia.

To shorten the total length of the capillary required all but about 3 mm. of the mercury column at the tip of the capillary are expelled by manipulation of the syringe. A column of double strength Van Slyke and Neill's reagent, of approximately the same length as the blood column, is then introduced into the capillary, between two mercury columns, in the manner described above.

Finally, a length of about 2 cm. of the capillary, containing the blood and reagent, is marked with a file and broken off through the mercury column. The lengths of the blood and reagent columns are measured, the ends of the capillary are sealed with De Khotinsky cement, and the blood and reagent are mixed by centrifuging. The appearance of the tube after sealing is shown in Fig. 2, *a*.

The air sample is prepared in the portion of the capillary tube adjacent to that used for containing the blood. To insure saturation of the air with water vapor, a column of water is placed between the mercury columns at the ends of the tube and each end of the column of air. The method of introduction of the air and water is similar to that already described for the blood and reagent. After several hours have been allowed for the air and water to come into equilibrium, the lengths of the columns are measured and the temperature is recorded. A 2 cm. length of the capillary is then marked with a file and broken off, and the ends of the tube are sealed with De Khotinsky cement (Fig. 2, *b*).

Since it was found that the observed galvanometer deflections are directly proportional, over a wide range, to the oxygen content of the samples used, the samples of blood and of air need not be prepared to yield the same amount of oxygen.

Preparation of Apparatus—After fresh reagents have been placed in the drying tube, the next step in the preparation of the apparatus for an experiment is to begin heating the mercury in the vapor pump. The potentials are usually applied to the amplifier at this time, since it requires about half an hour to become stable in operation. At the end of 15 or 20 minutes the Megavac pump is started and the filament chamber, the drying tube, and the chamber between them are connected to the pumps. The outside of the filament chamber is now heated carefully with a Bunsen flame for several minutes and then allowed to cool, at which time the gas pressure in the system should not be above 10^{-5} mm. of mercury. When the pressure has been reduced to this value, the tungsten filament is heated electrically to a cherry-red color. The filament temperature is unimportant, provided it is not too high, as will be discussed later. The current in the filament circuit is

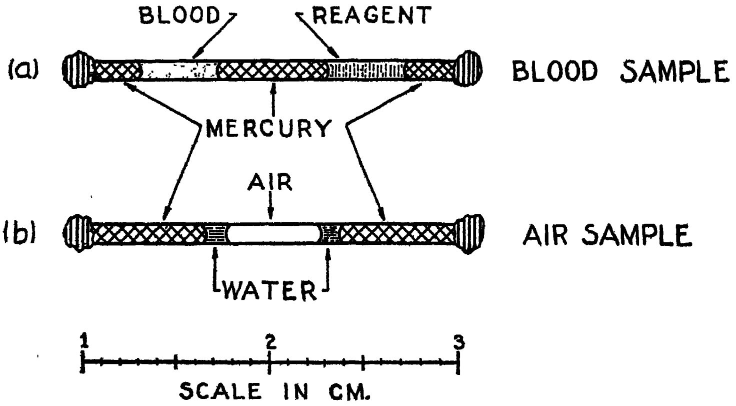


FIG. 2. Appearance of the sealed capillary tubes containing the samples.

measured by an ammeter and maintained constant to about 1 per cent by means of a variable resistance.

For about 15 minutes after the connection of the filament the reading of the galvanometer is found to be variable, owing to oxygen being given off from the internal surface of the filament chamber. When the galvanometer becomes constant, the tubes containing the samples to be analyzed are placed in the holes of plug *G* of Fig. 1 and the plug is seated in its sleeve. The filament chamber is then disconnected from the vacuum pumps, chambers *A* and *B* are evacuated, and the filament chamber is connected to the pumps again. The apparatus is now ready for the first determination. The preparations described, exclusive of renewing the drying agents, may be made in from 45 minutes to 1 hour.

Determination of Oxygen Content of a Sample—In order to clarify the procedure of a single determination, a schedule of the necessary steps is given below. Samples of blood and of air are analyzed alternately and with exactly the same procedure. In the cases in which the successive determinations on air fail to agree exactly, interpolation on the basis of time has been found to yield the expected accuracy for the estimations of blood oxygen.

When the apparatus has been prepared as described, it is regularly found that the sequence of manipulations outlined below gives rise to a galvanometer deflection even though no tube containing a sample has been broken. These blank estimations are repeated until the resulting galvanometer deflections reach a constant value, usually corresponding to approximately 0.005 c.mm. of oxygen, with a probable error of about ±0.001 c.mm. of oxygen. It is rarely necessary to make more than three or four blank estimations. The tube containing the first sample is then broken and the oxygen content of the sample determined, and a single blank estimation is made before breaking each subsequent tube. If the results of these blanks agree, it is certain that the apparatus does not leak.

The schedule for a determination is as follows:

1. At 0 minutes, 0 seconds, the hollow stop-cock *B* of Fig. 1 is placed in communication with the drying tube and stop-cock *F* is rotated through 180°, isolating chamber *A* from the vacuum system and breaking the capillary tube immediately below the projection *P*. (In the case of a blank determination, *F* is rotated

through only 90°, isolating chamber *A* from the pumps, but not breaking the capillary.)

2. At 45 seconds stop-cock *B* is turned to make connection with chamber *A*.

3. At 1 minute, 30 seconds, stop-cock *K* is closed and stop-cock *B* is opened to the drying tube *C*.

4. At 2 minutes, 30 seconds, stop-cock *L* is closed to disconnect the filament chamber from the vacuum pumps. Stop-cock *J* is closed to isolate a portion of the dried gases in chamber *D* and *K* is then opened, admitting this portion of the gases to the filament chamber. The maximum extent of the first deflection of the galvanometer, which occurs as soon as the oxygen molecules come into contact with the tungsten filament, is the reading recorded.

To prepare for another determination, the entire apparatus is again evacuated to a pressure of 10^{-5} mm. of mercury.

The times given above may be varied if desired. In several series of experiments, individual periods have been shortened as much as 50 per cent without significant change in the results. They may be lengthened indefinitely if the apparatus does not leak.

Following the determination of a series of samples contained in tubes in the holes of the glass plug *G*, the plug is unseated and all broken capillaries and dried blood are removed from the chamber *A*. The holes in *G* are then refilled with new samples, the plug is placed in position, chambers *A* and *B* are evacuated, and the apparatus is ready for another series of determinations.

Calculations and Correction of Results—The galvanometer deflection obtained from a sample was corrected by subtracting the deflection of the blank immediately preceding it.

Below is a sample calculation, from the experimental data given, of the oxygen content of a blood sample.

Air Sample—Length of air column, 3.00 mm.; total length of water columns, 1.00 mm.; deflection of blank, or zero correction, 4 mm.; deflection from sample, 166 mm.

Blood Sample—Length of blood column, 3.00 mm.; length of reagent column, 3.00 mm.; zero correction, 4 mm.; deflection from sample, 103 mm.

Equilibrations and measurements of column lengths were carried out at 23.0°, 760 mm. of mercury.

Zero Correction—Net deflection for air sample $= 166 - 4 = 162$ mm. Net deflection for blood sample $= 103 - 4 = 99$ mm.

Calculation of Galvanometer Deflection per Unit Length of Column of Dried Oxygen—The length of the column of dried oxygen in the air sample, reduced to standard conditions of temperature and pressure, is given by: $3.00 \times (760 - 21.1)/760 \times 273/(273 + 23) \times 0.2096 = 0.564$ mm. (saturation pressures of water vapor were taken from Landolt-Börnstein (1923)).

The corresponding value for the oxygen content of the distilled water in the air sample is given by: $1.00 \times 0.006 = 0.006$ mm.

The galvanometer deflection per unit length of column of dried oxygen under standard conditions, or the sensitivity of the apparatus, is: $162/0.570 = 284.1$ mm. of deflection per mm. of length.

Calculation of Oxygen Content of Blood Sample—Equivalent column of dried oxygen, under standard conditions, obtained from both the blood sample and the solution =

$$\frac{\text{Deflection for sample}}{\text{Sensitivity of apparatus}} = \frac{99}{284.1} = 0.348 \text{ mm.}$$

Column of dried oxygen, under standard conditions, obtained from solution alone $= 3.00 \times 0.006 = 0.018$ mm.

Column of dried oxygen, under standard conditions, obtained from blood alone $= 0.348 - 0.018 = 0.330$ mm.

Oxygen content of blood $= (100)(0.330)/3.00 = 11.0$ volumes per cent.

Control Experiments

Nitrogen and Carbon Dioxide—When allowance was made for the oxygen known to be present in dried tank nitrogen and carbon dioxide, it was found that these gases gave no deflection of the galvanometer when they were introduced into the chamber containing the hot tungsten filament. In other experiments it was found that neither nitrogen nor carbon dioxide diminished the accuracy of oxygen estimations when added in amounts up to 4 times the volume of the oxygen present.

When the filament current was increased by 50 per cent above the value necessary to give a cherry-red color to the filament, carbon dioxide still caused no decrease in the accuracy of the oxygen estimations. When the usual filament current was approximately doubled, however, the filament being hot enough to give a brilliant white light, it was found that the presence of carbon dioxide caused a decrease in the expected galvanometer deflections. For example, the response of the apparatus to oxygen was decreased approximately 20 per cent by the introduction of carbon dioxide and oxygen in equal amounts. This phenomenon was probably due to a chemical reaction which does not occur at the filament temperature ordinarily employed, but it has not been investigated further.

Water Vapor—Water vapor was found to give approximately 15 per cent of the deflection obtained for an equal volume of oxygen, but the passage of a gas saturated with water vapor through the drying tube described (chamber *C* of Fig. 1) prevented this effect. Thus it was found that samples containing air alone and samples containing both air and a column of water yielded the same galvanometer deflection per unit volume of oxygen, although the water present in the moistened samples was more than adequate to fill chambers *A* and *B* of Fig. 1 with water vapor at the tension corresponding to room temperature.

Method of Closing Tubes Containing Samples—Experiments were carried out in which samples of blood, prepared and sealed with De Khotinsky cement in the manner already described, were weighed before and after periods of centrifuging and exposure to a vacuum. In other experiments the oxygen gas contents of such samples were determined after varying periods of such treatment. No significant loss of contents was detected.

In order to determine the extent to which air might be included in the capillary tubes in an invisible form, and thus escape measurement, a series of thirteen tubes containing mercury alone was prepared. The average apparent oxygen content of the series was found to be 0.0014 ± 0.0007 c.mm. and in no case was more than 0.003 c.mm. of oxygen indicated.

Extraction Period—The adequacy of the extraction period of 90 seconds used in this investigation was demonstrated by a control experiment in which portions of the same blood sample were exposed to a vacuum for 90 seconds and for 15 seconds respectively. There was no significant difference in the amounts of oxygen given off in the two cases.

Reproducibility and Proportionality of Galvanometer Deflections—The reproducibility of the oxygen determinations and the constancy of the ratio of the electrical readings to the oxygen present were tested by the introduction of a series of twenty capillary tubes each containing approximately 1 c.mm. of air, and the introduction of another series of twelve samples containing from 0.1 to 4.0 c.mm. of air apiece. In each case the mean difference of the deflections per unit length of air column, from their average, was less than 2.5 per cent of the average deflection.

In order to determine the proportionality of the galvanometer deflections to the quantities of blood introduced, oxalated venous blood of man was confined under oil and a series of nine samples of the blood and potassium ferricyanide-saponin solution was prepared. With quantities of blood varying in volume from 0.3 to 1.5 c.mm. it was found that the deflections per unit length of sample showed no systematic variations and their mean difference from their average was 3.0 per cent of the average deflection.

Accuracy of Method

Determinations were made of the oxygen contents of oxalated venous bloods of man and of portions of these samples which had been equilibrated with air, the blood volumes of the samples being in all cases 1 c.mm. or less. The results were compared with determinations of portions of these bloods made by the method of Van Slyke and Neill (1924).[3] In a number of the experiments of this series, which are grouped separately in Table I, the carbon dioxide was removed from the gases extracted in the course of the microdeterminations by the addition of ascarite (Stetser, 1924) to the drying tube. It is evident that the presence of the carbon dioxide caused no significant systematic error.

Considering the entire series, the results by the method of Van Slyke and Neill have been compared with the respective oxygen contents as obtained by the micromethod. The values shown in Table I indicate that the results of determinations by the micromethod were, on the average, 0.16 volumes per cent lower than the oxygen contents yielded by the Van Slyke and Neill apparatus. This difference may be caused by incomplete extraction of the oxygen from the blood.

The individual results by the micromethod were corrected for the systematic error and the average deviations of the results from the respective Van Slyke and Neill values were determined. The mean deviation found indicated an average non-systematic error for a single determination of 0.56 volume per cent.

---

[3] Dr. L. H. Collins and Mrs. C. L. Allison were so kind as to offer their services in making the Van Slyke and Neill determinations.

TABLE I

Comparison of Blood Oxygen Determinations Made by Micromethod and by Method of Van Slyke and Neill

All data are expressed in volumes per cent.

Van Slyke-Neill method	Micromethod (CO_2 not removed from extracted gases)		Van Slyke-Neill method	Micromethod (CO_2 removed from extracted gases)	
	Individual determinations	Average O_2 content		Individual determinations	Average O_2 content
19.4	19.0, 18.8, 19.5, 19.2	19.1	15.0	14.2, 14.3, 14.0	14.2
19.5	19.7, 20.0, 19.5, 19.0, 18.8, 17.9	19.2	14.5	14.0, 14.4, 14.7, 14.0, 14.8, 14.7	14.5
8.7	8.1, 9.0, 7.9, 8.2	8.3	7.7	7.2, 7.6, 7.3, 6.7	7.1
8.7	8.6, 8.0, 9.5	8.7	7.8	7.9, 7.6, 7.5, 7.7	7.7
6.5	7.0, 6.3, 6.6, 6.5	6.6	8.3	8.1, 8.6, 10.0, 8.9, 6.4	8.2
14.1	12.5, 14.3, 14.6	13.8	11.4	10.5, 10.9, 10.6, 11.3, 11.6, 10.4	10.9
17.8	18.4, 17.8, 17.0	17.7			
9.7	9.1, 9.6, 9.1	9.3	11.4	10.2, 11.9, 12.8	11.6
11.0	10.8, 12.6, 10.6	11.3	11.9	11.7, 12.4, 11.4, 13.0, 11.2, 11.7, 11.2	11.7
15.0	14.5, 14.0, 15.6, 15.3	14.8			
12.8	11.3, 12.2, 12.8	12.1	6.7	6.1, 8.2, 7.6, 6.3	7.0
17.6	17.0, 18.3, 18.5	17.9			
15.8	14.7, 14.8, 15.0	14.6			

The average systematic difference, for the entire series, is −0.16 volume per cent. After correction for the systematic difference, the average non-systematic difference for the entire series is 0.56 volume per cent.

SUMMARY

1. An electrical method is described for the determination of oxygen gas in amounts less than 1 c.mm. The positive ion current, resulting from the action of the oxygen upon a hot tungsten filament, was amplified and passed through a sensitive galvanometer. The initial galvanometer deflections were found to be proportional to the oxygen in the samples used.

2. Determinations are shown to be unaffected by the presence of nitrogen, carbon dioxide, and water vapor.

3. A technique is described for the application of the method to the determination of the oxygen content of samples of blood of 1 c.mm. in volume.

4. The results of a series of blood oxygen determinations on 1 c.mm. samples of blood were found to be, on the average, 0.16 volume per cent lower than the results of determinations on 1 cc. samples of the same bloods by the method of Van Slyke and Neill.

5. In the same series of determinations the values obtained by the micromethod were corrected for the systematic difference and the average deviation of the results from the Van Slyke and Neill values was found to be 0.56 volume per cent.

I make grateful acknowledgment of fruitful suggestions and valuable help from Professors A. N. Richards and D. W. Bronk, and Drs. A. M. Walker and C. J. Gamble.

BIBLIOGRAPHY

Donal, J. S., Jr., *Physical Rev.*, **36**, 1172 (1930).
Hartridge, H., *J. Physiol.*, **44**, 1 (1912).
Krogh, A., *J. Physiol.*, **52**, 281 (1919).
Laws, F. A., Electrical measurements, New York, 117 (1917).
Mook, H. W., *Biochem. Z.*, **223**, 152 (1930); **242**, 338 (1931).
Razek, J., and Mulder, P. J., *J. Opt. Soc. Am.*, **19**, 390 (1929).
Richards, A. N., Bordley, J., 3rd, and Walker, A. M., *J. Biol. Chem.*, **101**, 179 (1933).
Roth, W. A., and Scheel, K., Landolt-Börnstein physikalisch-chemische Tabellen, Berlin, 5th edition, **1** (1923).
Stetser, J. B., *Chem. and Ind.*, **43**, 637 (1924).
Van Slyke, D. D., *J. Biol. Chem.*, **30**, 347 (1917).
Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
Willard, H. H., and Smith, G. F., *J. Am. Chem. Soc.*, **44**, 2255 (1922).

INDEX TO AUTHORS

A

Almaden, Phillip. See Yavorsky, Almaden, and King, 525

Almquist, H. J., Lorenz, F. W., and Burmester, Ben R. Relation of depot fat to egg yolk fat in laying hens, 365

B

Babers, Frank H. See Goebel and Babers, 63

Baernstein, Harry D. A modification of the method for determining methionine in proteins, 451

Ball, Eric G. Studies on oxidation-reduction. XXI. Phthiocol, the pigment of the human tubercle bacillus, 515

Balls, A. K., and Swenson, T. L. The antitrypsin of egg white, 409

Barbour, A. D. The effect of the saturated fatty acid content of the diet on the composition of the body fat, 281

Bauguess, Lyle C., and Berg, Clarence P. Tryptophane metabolism. VII. Growth and kynurenic acid production on amides of *l*-tryptophane, 615

Berg, Clarence P. See Bauguess and Berg, 615

Bernheim, Frederick, and Bernheim, Mary L. C. The oxidation of proline and alanine by certain tissues, 79

Bernheim, Mary L. C. See Bernheim and Bernheim, 79

Block, Richard J. The determination of the basic amino acids in small quantities of proteins by the silver precipitation method, 457

Bosworth, A. W. Studies of the fat of human milk, 235

Burmester, Ben R. See Almquist, Lorenz, and Burmester, 365

Butler, Allan M., and MacKay, Eaton M. The influence of the sodium and potassium content of the diet upon the sodium concentration of human centrifuged red blood cells, 107

C

Chaikoff, I. L., and Kaplan, A. The blood lipids in completely depancreatized dogs maintained with insulin, 267

Chase, Barbara W., and Lewis, Howard B. Comparative studies of the metabolism of amino acids. VI. The rate of absorption of leucine, valine, and their isomers from the gastrointestinal tract of the white rat, 315

Clarke, H. T. See Fruton and Clarke, 667

Craig, Lyman C. See Jacobs and Craig, 393

Cullen, Glenn E. See Robinson, Price, and Cullen, 7

Cutler, Jessie T. Studies on the carbohydrate metabolism of the goat. The blood sugar and the inorganic phosphate, 653

D

Dennett, Linnea. See Halliday, 29

Doisy, Edward A. See Katzman and Doisy, 125

Donal, John S., Jr. An ultramicromethod for the determination of the oxygen content of blood, 783

Dyer, Helen M. See du Vigneaud, Dyer, Jones, and Patterson, 401

E

Eck, John C., and Marvel, Carl S. A convenient synthesis of *dl*-lysine, 387

Elderfield, Robert C., and Rothen, Alexandre. Strophanthin. XXX. The ultra-violet absorption spectra of trianhydrostrophanthidin and trianhydroperiplogenin derivatives, 71

Elvehjem, C. A. See Phillips, Stare, and Elvehjem, 41

—. See Schultze, Elvehjem, and Hart, 735

Erickson, Betty Nims, Gulick, Margaret, Hunscher, Helen A., and Macy, Icie G. Human milk studies. XV. The non-protein nitrogen constituents, 145

Evans, Herbert M., Lepkovsky, Samuel, and Murphy, Elizabeth A. Vital need of the body for certain unsaturated fatty acids. IV. Reproduction and lactation upon fat-free diets, 431

—, —, and —. V. Reproduction and lactation upon diets containing saturated fatty acids as their sole source of energy, 441

—, —, and —. VI. Male sterility on fat-free diets, 445

F

Fisher, A. M., and Scott, D. A. The insulin content of the pancreas in cattle of various ages, 305

— and —. Peptic hydrolysis of insulin, 289

Folin, Otto. The preparation of sodium tungstate free from molybdate, together with a simplified process for the preparation of a correct uric acid reagent (and some comments), 311

Fruton, J. S., and Clarke, H. T. Chemical reactivity of cystine and its derivatives, 667

G

Gersdorff, Charles E. F. See Jones and Gersdorff, 707

Goddard, David R., and Michaelis, Leonor. A study on keratin, 605

Goebel, Walther F., and Babers, Frank H. Derivatives of glucuronic acid. IV. The synthesis of α- and β-tetraacetylglucuronic acid methyl ester and of 1-chlorotriacetylglucuronic acid methyl ester, 63

Green, Arda Alden. See Root and Green, 545

Gulick, Margaret. See Erickson, Gulick, Hunscher, and Macy, 145

H

Halliday, Nellie. Further investigations concerning the new vitamin B growth-promoting factor for rats, found in whole wheat, 29

Hanner, James P. See Wilson and Hanner, 323

Hart, E. B. See Schultze, Elvehjem, and Hart, 735

Heller, V. G., Paul, Henry, and Thompson, R. B. Changes in the blood calcium and phosphorus partition during the life cycle of the chicken, 357

Hilbert, Guido E., and Jansen, Eugene F. A study of the absorption spectra of some carotenoid pigments at liquid air temperatures and its applications to the carotenoid pigments of cow-pea leaves (*Vigna sinensis*), 97

Holm, George E. See Kurtz, Jamieson, and Holm, 717

Hunscher, Helen A. See Erickson, Gulick, Hunscher, and Macy, 145

J

Jacobs, Walter A., and Craig, Lyman C. The ergot alkaloids. III. On lysergic acid, 393

Jamieson, G. S. See Kurtz, Jamieson, and Holm, 717

Jansen, Eugene F. See Hilbert and Jansen, 97

Jones, Chase B. See du Vigneaud, Dyer, Jones, and Patterson, 401

Jones, D. Breese, and Gersdorff, Charles E. F. Studies on digestibility of proteins *in vitro*. VI. Some partial cleavage products from peptic digests of casein, 707

Jones, James H. The influence of the removal of the parathyroid glands on the development of rickets in rats, 701

K

Kaplan, A. See Chaikoff and Kaplan, 267

Katzman, Philip A., and Doisy, Edward A. The quantitative determination of small amounts of gonadotropic material, 125

Keil, H. L., and Nelson, Victor E. The rôle of copper in carbohydrate metabolism, 343

Kimmel, Louise. See Morgan, Kimmel, Thomas, and Samisch, 531

King, C. G. See Yavorsky, Almaden, and King, 525

Kirk, Esben. Gasometric microdetermination of phosphoric acid, 191

—, Page, Irvine H., and Van Slyke, Donald D. Gasometric microdetermination of lipids in plasma, blood cells, and tissues, 203

Kleiner, Israel S., and Tauber, Henry. Further studies on the zymogens of pepsin and rennin, 501

Kruse, H. D., Schmidt, Marguerite M., and McCollum, E. V. Studies on magnesium deficiency in animals. V. Changes in the mineral metabolism of animals following magnesium deprivation, 553

—. See Orent, Kruse, and McCollum, 573

Kulchar, George V. See Pillsbury and Kulchar, 351

Kurtz, Floyd E., Jamieson, G. S., and Holm, George E. The lipids of milk. I. The fatty acids of the lecithin-cephalin fraction, 717

L

Lamson, Paul D. See Robbins and Lamson, 725

Laug, Edwin P. A reinvestigation of the phenomenon of a first acid change in whole blood, 161

Lepkovsky, Samuel. See Evans, Lepkovsky, and Murphy, 431, 441, 445

Letonoff, T. V. A zinc hydroxide powder for the preparation of protein-free filtrates of blood, 693

Levene, P. A., and Marker, R. E. Symmetrical disubstituted methanes prepared from members of optically active homologous series of disubstituted carboxylic acids and their derivatives, 173

— **and Muskat, Irving E.** The synthesis of theophylline-5-methyl-rhamnofuranoside, 761

Levene, P. A., and Schormüller, A. Synthesis of the phosphoric esters of hydroxyamino acids. III. Resolution of *dl*-serinephosphoric acid and synthesis of *l*-hydroxyprolinephosphoric acid, 595

— **and Stiller, Eric T.** Acetone derivatives of *d*-ribose. II, 421

— **and Tipson, R. Stuart.** Monoacetone *d*-xyloketose. A preliminary note, 603

— and —. The partial synthesis of ribose nucleotides. I. Uridine 5-phosphoric acid, 113

Lewis, Howard B. See Chase and Lewis, 315

Link, Karl Paul. See Niemann and Link, 773

Lorenz, F. W. See Almquist, Lorenz, and Burmester, 365

M

MacKay, Eaton M. See Butler and MacKay, 107

Macy, Icie G. See Erickson, Gulick, Hunscher, and Macy, 145

Marker, R. E. See Levene and Marker, 173

Marvel, Carl S. See Eck and Marvel, 387

McCollum, E. V. See Kruse, Schmidt, and McCollum, 553

—. See Orent, Kruse, and McCollum, 573

McFarlane, W. D. The distribution of iron in tissues, particularly liver, during peptic digestion and autolysis, 245

Michaelis, Leonor, and Schubert, Maxwell P. The reaction of iodoacetic acid on mercaptans and amines, 331

—. See GODDARD and MICHAELIS, 605

Morgan, Agnes Fay, Kimmel, Louise, Thomas, Rachel, and Samisch, Zdenka. The effects of moderate doses of viosterol and of parathyroid extract upon rats, 531

Murphy, Elizabeth A. See EVANS, LEPKOVSKY, and MURPHY, 431, 441, 445

Muskat, Irving E. See LEVENE and MUSKAT, 761

N

Nelson, Victor E. See KEIL and NELSON, 343

Niemann, Carl, and Link, Karl Paul. Synthesis of the hexuronic acids. VII. The synthesis of *l*-glucuronic acid and the resolution of *dl*-galacturonic acid, 773

O

Orent, Elsa R., Kruse, H. D., and McCollum, E. V. Studies on magnesium deficiency in animals. VI. Chemical changes in the bone, with associated blood changes, resulting from magnesium deprivation, 573

P

Page, Irvine H. See KIRK, PAGE, and VAN SLYKE, 203

Patterson, Wilbur I. See DU VIGNEAUD, DYER, JONES, and PATTERSON, 401

Paul, Henry. See HELLER, PAUL, and THOMPSON, 357

Pearson, P. B. Inorganic phosphorus of horse serum. The effect of age and nutrition, 1

Pfiffner, J. J., and Vars, Harry M. Extraction studies on the adrenal cortical hormone. III. Distribution studies, 645

—, —, **and Taylor, A. R.** Extraction studies on the adrenal cortical hormone. I. Methods of preparation, 625

—. See VARS, TAYLOR, and PFIFFNER, 639

Phillips, Paul H., Stare, F. J., and Elvehjem, C. A. A study of tissue respiration and certain reducing substances in chronic fluorosis and scurvy in the guinea pig, 41

Pillsbury, Donald M., and Kulchar, George V. The use of the Hagedorn-Jensen method in the determination of skin glucose, 351

Platt, Muriel E., and Schroeder, E. F. Glyoxalase. II. The distribution of glyoxalase in tissues of normal and cancerous albino rats, 179

Price, J. Waide. See ROBINSON, PRICE, and CULLEN, 7

R

Robbins, Benjamin H., and Lamson, Paul D. Further studies on the proteolytic enzyme content of latex from the fig and related trees, 725

Robinson, Howard W., Price, J. Waide, and **Cullen, Glenn E.** Studies of the acid-base condition of blood. III. The value of pK′ in the Henderson-Hasselbalch equation for human and dog sera, determined with the Simms electrode, 7

Root, Raymond W., and **Green, Arda Alden.** The effect of acidity on the carbon monoxide-combining power of hemoglobin in the blood of marine fishes, 545

Rothen, Alexandre. See Elderfield and Rothen, 71

Russell, Mary A. See Weil and Russell, 505

S

Samisch, Zdenka. See Morgan, Kimmel, Thomas, and Samisch, 531

Schmidt, Marguerite M. See Kruse, Schmidt, and McCollum, 553

Schoenheimer, Rudolf, and **Sperry, Warren M.** A micromethod for the determination of free and combined cholesterol, 745

Schormüller, A. See Levene and Schormüller, 595

Schroeder, E. F. See Platt and Schroeder, 179

Schubert, Maxwell P. See Michaelis and Schubert, 331

Schultze, M. O., Elvehjem, C. A., and **Hart, E. B.** The availability of copper in various compounds as a supplement to iron in hemoglobin formation, 735

Scott, D. A. See Fisher and Scott, 289, 305

Seelig, S. See Sendroy, Seelig, and Van Slyke, 463, 479

Seibert, Florence B. See Spiegel-Adolf and Seibert, 373

Sendroy, Julius, Jr., Seelig, S., and **Van Slyke, Donald D.** Studies of acidosis. XXII. Application of the Henderson-Hasselbalch equation to human urine, 463

—, —, and —. XXIII. The carbon dioxide tension and acid-base balance of human urine, 479

Sperry, Warren M. See Schoenheimer and Sperry, 745

Spiegel-Adolf, Mona, and **Seibert, Florence B.** Spectral analysis of purified tuberculin, 373

Spielman, M. A. The chemistry of the lipids of tubercle bacilli. XXXIX. The constitution of tuberculostearic acid, 87

Stare, F. J. See Phillips, Stare, and Elvehjem, 41

Stiller, Eric T. See Levene and Stiller, 421

Swenson, T. L. See Balls and Swenson, 409

T

Tauber, Henry. See Kleiner and Tauber, 501

Taylor, A. R. See Pfiffner, Vars, and Taylor, 625

—. See Vars, Taylor, and Pfiffner, 639

Ten Broeck, Carl. The differentiation of trypsins by means of the anaphylactic test, 729

Thomas, Rachel. See Morgan, Kimmel, Thomas, and Samisch, 531

Thompson, R. B. See Heller, Paul, and Thompson, 357

Tipson, R. Stuart. See Levene and Tipson, 113, 603

V

Van Slyke, Donald D. See Kirk, Page, and Van Slyke, 203

—. See Sendroy, Seelig, and Van Slyke, 463, 479

Vars, Harry M., Taylor, A. R., and **Pfiffner, J. J.** Extraction studies on the adrenal cortical hormone. II. Yield from glands of various species, 639

—. See Pfiffner and Vars, 645

—. See Pfiffner, Vars, and Taylor, 625

du Vigneaud, Vincent, Dyer, Helen M., Jones, Chase B., and **Patterson, Wilbur I.** The synthesis of pentocystine and homomethionine, 401

W

Weil, Leopold, and **Russell, Mary A.** A manometric micromethod for arginase determination. Enzymatic study of blood arginase in rats, 505

White, Julius. The synthesis of cystinyldiglycine and cystinyldialanine, 141

Wilson, William R., and **Hanner, James P.** Changes of total lipid and iodine number of blood fat in alimentary lipemia, 323

Y

Yavorsky, Martin, Almaden, Phillip, and **King, C. G.** The vitamin C content of human tissues, 525

INDEX TO SUBJECTS

A

Acid: Blood, first change (Laug) 161

Acid-base: Blood (Robinson, Price, and Cullen) 7

Acid-base equilibrium: Urine (Sendroy, Seelig, and Van Slyke) 479

Acidosis: (Sendroy, Seelig, and Van Slyke) 463, 479

Adrenals: Cortex hormone, distribution (Pfiffner and Vars) 645
  — —, preparation (Pfiffner, Vars, and Taylor) 625
  — —, various species (Vars, Taylor, and Pfiffner) 639

Age: Blood serum inorganic phosphorus, horse, effect (Pearson) 1
  Pancreas insulin, cattle, effect (Fisher and Scott) 305

Alanine: Cystinyldi-, synthesis (White) 141
  Oxidation, tissues (Bernheim and Bernheim) 79

Alkaloids: Ergot (Jacobs and Craig) 393

Amides: *l*-Tryptophane, growth and kynurenic acid production, effect (Bauguess and Berg) 615

Amines: Iodoacetic acid reaction (Michaelis and Schubert) 331

Amino acids: Basic, proteins, determination (Block) 457
  Hydroxy-, phosphoric esters, synthesis (Levene and Schormüller) 595
  Metabolism (Chase and Lewis) 315

Antitrypsin: Egg white (Balls and Swenson) 409

Arginase: Blood (Weil and Russell) 505
  Microdetermination, manometric (Weil and Russell) 505

B

Blood: Acid change, first (Laug) 161
  Acid-base (Robinson, Price, and Cullen) 7
  Filtrates, protein-free, zinc hydroxide powder for preparation (Letonoff) 693
  Lipid, total, lipemia, alimentary (Wilson and Hanner) 323
  Lipids, depancreatized dog, insulin effect (Chaikoff and Kaplan) 267
  Magnesium deprivation effect (Orent, Kruse, and McCollum) 573

Blood cell: Lipids, microdetermination, gasometric (Kirk, Page, and Van Slyke) 203

Blood plasma: Lipids, microdetermination, gasometric (Kirk, Page, and Van Slyke) 203

Blood serum: Dissociation constant, apparent, Henderson-Hasselbalch equation (Robinson, Price, and Cullen) 7

Blood sugar: Goat (Cutler) 653

Bone: Magnesium deprivation effect (Orent, Kruse, and McCollum) 573

C

Calcium: Blood, partition, chicken (Heller, Paul, and Thompson) 357

Cancer: Tissue, glyoxalase (Platt and Schroeder) 179

Carbohydrate: Metabolism, copper rôle (Keil and Nelson) 343

—, goat (Cutler) 653

Carbon dioxide: Urine, tension (Sendroy, Seelig, and Van Slyke) 479

Carbon monoxide: -Combining power, blood hemoglobin, fish, acidity effect (Root and Green) 545

Carboxylic acids: Disubstituted, symmetrical disubstituted methanes from optically active homologous series (Levene and Marker) 173

Carotenoid pigments: Absorption spectra, liquid air temperatures (Hilbert and Jansen) 97

Carotenoid pigments—*continued:*
Cow-pea leaves, absorption spectra, liquid air temperatures (Hilbert and Jansen) 97

Casein: Peptic digest (Jones and Gersdorff) 707

Cephalin: Lecithin-, fraction, milk, fatty acids (Kurtz, Jamieson, and Holm) 717

Chicken: Blood calcium and phosphorus, partition (Heller, Paul, and Thompson) 357

1-Chlorotriacetylglucuronic acid: Methyl ester, synthesis (Goebel and Babers) 63

Cholesterol: Free and combined, microdetermination (Schoenheimer and Sperry) 745

Copper: Carbohydrate metabolism, rôle (Keil and Nelson) 343
Hemoglobin formation, iron supplement (Schultze, Elvehjem, and Hart) 735

Cow-pea: *See* Pea

Cystine: Derivatives, reactivity (Fruton and Clarke) 667
Pento-, synthesis (du Vigneaud, Dyer, Jones, and Patterson) 401
Reactivity (Fruton and Clarke) 667

Cystinyldialanine: Synthesis (White) 141

Cystinyldiglycine: Synthesis (White) 141

E

Egg: White, antitrypsin (Balls and Swenson) 409
  Yolk fat, depot fat, relation (Almquist, Lorenz, and Burmester) 365
Enzyme: Proteolytic, fig tree latex (Robbins and Lamson) 725
  See also Antitrypsin, Arginase, Glyoxalase, Pepsin, Rennin, Trypsin
Ergot: Alkaloids (Jacobs and Craig) 393

F

Fat: Blood, lipemia, alimentary, effect (Wilson and Hanner) 323
  Body, fatty acids, saturated, dietary, effect (Barbour) 281
  Egg yolk, depot fat, relation (Almquist, Lorenz, and Burmester) 365
  -Free diet, male sterility effect (Evans, Lepkovsky, and Murphy) 445
  — —, reproduction and lactation, effect (Evans, Lepkovsky, and Murphy) 431
  Milk, human (Bosworth) 235
Fatty acids: Milk, lecithin-cephalin fraction (Kurtz, Jamieson, and Holm) 717
  Saturated, as energy source, reproduction and lactation effect (Evans, Lepkovsky, and Murphy) 441
  —, dietary, body fat, effect (Barbour) 281
Fatty acids—*continued:*
  Unsaturated, vital need (Evans, Lepkovsky, and Murphy) 431, 441, 445
Fig: Tree, latex, proteolytic enzyme (Robbins and Lamson) 725
Fish: Blood hemoglobin carbon monoxide-combining power, acidity effect (Root and Green) 545
Fluorosis: Reducing substances (Phillips, Stare, and Elvehjem) 41
  Tissue respiration (Phillips, Stare, and Elvehjem) 41

G

Galacturonic acid: *dl*-, resolution (Niemann and Link) 773
Glucose: Skin, determination, Hagedorn-Jensen method (Pillsbury and Kulchar) 351
Glucuronic acid: α- and β-tetraacetyl-, methyl ester, synthesis (Goebel and Babers) 63
  1-Chlorotriacetyl-, methyl ester, synthesis (Goebel and Babers) 63
  Derivatives (Goebel and Babers) 63
  l-, synthesis (Niemann and Link) 773
Glycine: Cystinyldi-, synthesis (White) 141
Glyoxalase: Tissue, normal and cancerous (Platt and Schroeder) 179
Goat: Blood phosphate, inorganic (Cutler) 653
  — sugar (Cutler) 653

Gonadotropic material: Determination (KATZMAN and DOISY) 125

Growth: *l*-Tryptophane amides, effect (BAUGUESS and BERG) 615

H

Hagedorn-Jensen: Skin glucose, determination (PILLSBURY and KULCHAR) 351

Hemoglobin: Blood, carbon monoxide-combining power, fish, acidity effect (ROOT and GREEN) 545

  Formation, copper supplement to iron (SCHULTZE, ELVEHJEM, and HART) 735

Henderson-Hasselbalch: Equation, blood serum, apparent dissociation constant, value (ROBINSON, PRICE, and CULLEN) 7

  —, urine, application (SENDROY, SEELIG, and VAN SLYKE) 463

Hexuronic acids: Synthesis (NIEMANN and LINK) 773

Homomethionine: Synthesis (DU VIGNEAUD, DYER, JONES, and PATTERSON) 401

Hydroxyamino acids: Phosphoric esters, synthesis (LEVENE and SCHORMÜLLER) 595

Hydroxyprolinephosphoric acid: *l*-, synthesis (LEVENE and SCHORMÜLLER) 595

I

Insulin: Blood lipids, depancreatized dog, effect (CHAIKOFF and KAPLAN) 267

Insulin—*continued:*

  Pancreas, cattle, age effect (FISHER and SCOTT) 305

  Peptic hydrolysis (FISHER and SCOTT) 289

Iodoacetic acid: Mercaptans and amines, reaction (MICHAELIS and SCHUBERT) 331

Iron: Hemoglobin formation, copper supplement (SCHULTZE, ELVEHJEM, and HART) 735

  Liver, distribution, peptic digestion and autolysis (MCFARLANE) 245

  Tissue, distribution, peptic digestion and autolysis (MCFARLANE) 245

K

Keratin: (GODDARD and MICHAELIS) 605

Kynurenic acid: Production, *l*-tryptophane amides, effect (BAUGUESS and BERG) 615

L

Lactation: Fat-free diet, effect (EVANS, LEPKOVSKY, and MURPHY) 431

  Fatty acids, saturated, as energy source (EVANS, LEPKOVSKY, and MURPHY) 441

Latex: Fig tree, proteolytic enzyme in (ROBBINS and LAMSON) 725

Lecithin: -Cephalin fraction, milk, fatty acids (KURTZ, JAMIESON, and HOLM) 717

Leucine: Absorption rate, gastrointestinal tract (CHASE and LEWIS) 315

Leucine—*continued:*
  Isomers, absorption rate, gastrointestinal tract (Chase and Lewis) 315

Lipemia: Alimentary, blood lipid, total, and iodine number, effect (Wilson and Hanner) 323

Lipid(s): Blood cells, microdetermination, gasometric (Kirk, Page, and Van Slyke) 203
  —, depancreatized dog, insulin effect (Chaikoff and Kaplan) 267
  — plasma, microdetermination, gasometric (Kirk, Page, and Van Slyke) 203
  Milk (Kurtz, Jamieson, and Holm) 717
  Tissue, microdetermination, gasometric (Kirk, Page, and Van Slyke) 203
  Total, blood, lipemia, alimentary, effect (Wilson and Hanner) 323
  Tubercle bacillus, chemistry (Spielman) 87

Liver: Iron distribution, peptic digestion and autolysis (McFarlane) *245

Lysergic acid: (Jacobs and Craig) 393

Lysine: *dl*-, synthesis (Eck and Marvel) 387

M

Magnesium: Deficiency (Kruse, Schmidt, and McCollum) 553
  (Orent, Kruse, and McCollum) 573

Magnesium—*continued:*
  Deprivation, blood effect (Orent, Kruse, and McCollum) 573
  —, bone effect (Orent, Kruse, and McCollum) 573
  —, mineral metabolism effect (Kruse, Schmidt, and McCollum) 553

Mercaptans: Iodoacetic acid reaction (Michaelis and Schubert) 331

Methanes: Disubstituted, symmetrical, from optically active homologous series of disubstituted carboxylic acids and derivatives (Levene and Marker) 173

Methionine: Homo-, synthesis (du Vigneaud, Dyer, Jones, and Patterson) 401
  Proteins, determination (Baernstein) 451

Milk: Human, non-protein nitrogen constituents (Erickson, Gulick, Hunscher, and Macy) 145
  Lipids (Kurtz, Jamieson, and Holm) 717

Mineral: Metabolism, magnesium deprivation, effect (Kruse, Schmidt, and McCollum) 553

Monoacetone *d*-**xyloketose:** (Levene and Tipson) 603

N

Nitrogen: Non-protein, constituents, milk, human (Erickson, Gulick, Hunscher, and Macy) 145

Nucleotides: Ribose, synthesis (Levene and Tipson) 113

Nutrition: Blood serum inorganic phosphorus, horse, effect (Pearson) 1

O

Oxidation-reduction: (Ball) 515

Oxygen: Blood, ultramicrodetermination (Donal) 783

P

Pancreas: Insulin, cattle, age effect (Fisher and Scott) 305

Pancreatectomy: Blood lipids, insulin effect (Chaikoff and Kaplan) 267

Parathyroid extract: (Morgan, Kimmel, Thomas, and Samisch) 531

Parathyroidectomy: Rickets, effect (Jones) 701

Pentocystine: Synthesis (du Vigneaud, Dyer, Jones, and Patterson) 401

Pepsin: Casein digestion (Jones and Gersdorff) 707

  Digestion, tissues, iron in (McFarlane) 245

  Insulin hydrolysis (Fisher and Scott) 289

  Zymogens (Kleiner and Tauber) 501

Periplogenin: Trianhydro-, derivatives, ultra-violet absorption spectra (Elderfield and Rothen) 71

Phosphate: Inorganic, blood, goat (Cutler) 603

Phosphoric acid: Microdetermination, gasometric (Kirk) 191

Phosphorus: Blood, partition, chicken (Heller, Paul, and Thompson) 357

  Inorganic, blood serum, age and nutrition, effect (Pearson) 1

Phthiocol: Tubercle bacillus, human (Ball) 515

Pigment: Carotenoid, absorption spectra, liquid air temperatures (Hilbert and Jansen) 97

  —, cow-pea leaves, absorption spectra, liquid air temperatures (Hilbert and Jansen) 97

  Tubercle bacillus, human (Ball) 515

Potassium: Dietary, blood cell, red, sodium, influence (Butler and MacKay) 107

Proline: Oxidation, tissues (Bernheim and Bernheim) 79

Prolinephosphoric acid: *l*-Hydroxy-, synthesis (Levene and Schormüller) 595

Proteins: Amino acids, basic, determination (Block) 457

  Digestibility *in vitro* (Jones and Gersdorff) 707

  Methionine determination (Baernstein) 451

Proteolysis: Enzyme, fig tree latex (Robbins and Lamson) 725

R

Reducing substances: Fluorosis (Phillips, Stare, and Elvehjem) 41

  Scurvy (Phillips, Stare, and Elvehjem) 41

Rennin: Zymogens (KLEINER and TAUBER) 501
Reproduction: Fat-free diet, effect (EVANS, LEPKOVSKY, and MURPHY) 431
  Fatty acids, saturated, as energy source (EVANS, LEPKOVSKY, and MURPHY) 441
Rhamnofuranoside: Theophylline-5-methyl-, synthesis (LEVENE and MUSKAT) 761
Ribose: *d*-, acetone derivatives (LEVENE and STILLER) 421
  Nucleotides, synthesis (LEVENE and TIPSON) 113
Rickets: Parathyroidectomy effect (JONES) 701

S

Scurvy: Reducing substances (PHILLIPS, STARE, and ELVEHJEM) 41
  Tissue respiration (PHILLIPS, STARE, and ELVEHJEM) 41
Serinephosphoric acid: *dl*-, resolution (LEVENE and SCHORMÜLLER) 595
Skin: Glucose, determination, Hagedorn-Jensen method (PILLSBURY and KULCHAR) 351
Sodium: Blood cell, red, dietary sodium and potassium, influence (BUTLER and MACKAY) 107
  Dietary, blood cell, red, sodium, influence (BUTLER and MACKAY) 107
Sodium tungstate: Molybdate-free, preparation (FOLIN) 311
Sterility: Male, fat-free diet effect (EVANS, LEPKOVSKY, and MURPHY) 445
Strophanthidin: Trianhydro-, derivatives, ultra-violet absorption spectra (ELDERFIELD and ROTHEN) 71
Strophanthin: (ELDERFIELD and ROTHEN) 71

T

Tetraacetylglucuronic acid: α- and β-, methyl ester, synthesis (GOEBEL and BABERS) 63
Theophylline-5-methyl-rhamnofuranoside: Synthesis (LEVENE and MUSKAT) 761
Tissue: Alanine oxidation by (BERNHEIM and BERNHEIM) 79
  Lipids, microdetermination, gasometric (KIRK, PAGE, and VAN SLYKE) 203
  Proline oxidation by (BERNHEIM and BERNHEIM) 79
  Respiration, fluorosis and scurvy (PHILLIPS, STARE, and ELVEHJEM) 41
Trianhydroperiplogenin: Derivatives, ultra-violet absorption spectra (ELDERFIELD and ROTHEN) 71
Trianhydrostrophanthidin: Derivatives, ultra-violet absorption spectra (ELDERFIELD and ROTHEN) 71
Tryspin: Anti-, egg white (BALLS and SWENSON) 409
  Differentiation, anaphylactic test (TEN BROECK) 729

Tryptophane: *l*-, amides, growth effect (Bauguess and Berg) 615

—, —, kynurenic acid production (Bauguess and Berg) 615

Metabolism (Bauguess and Berg) 615

Tubercle bacillus: Human, phthiocol (Ball) 515

Lipids, chemistry (Spielman) 87

Tuberculostearic acid (Spielman) 87

Tuberculin: Purified, spectrum analysis (Spiegel-Adolf and Seibert) 373

Tuberculostearic acid: Tubercle bacillus lipids (Spielman) 87

U

Uric acid: Reagent, preparation (Folin) 311

Uridine-5-phosphoric acid: Synthesis (Levene and Tipson) 113

Urine: Acid-base equilibrium (Sendroy, Seelig, and Van Slyke) 479

Carbon dioxide tension (Sendroy, Seelig, and Van Slyke) 479

Henderson-Hasselbalch equation, application (Sendroy, Seelig, and Van Slyke) 463

V

Valine: Absorption rate, gastrointestinal tract (Chase and Lewis) 315

Isomers, absorption rate gastrointestinal tract (Chase and Lewis) 315

Vigna sinensis: *See* Pea

Viosterol: (Morgan, Kimmel, Thomas, and Samisch) 531

Vitamin: B growth-promoting factor, whole wheat (Halliday) 29

C, tissue, human (Yavorsky, Almaden, and King) 525

W

Wheat: Whole, vitamin B growth-promoting factor (Halliday) 29

X

Xyloketose: *d*-, monoacetone (Levene and Tipson) 603

Z

Zinc hydroxide: Blood filtrates, protein-free, preparation (Letonoff) 693